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THE UNIVERSITY OF ALBERTA

SEX HORMONES IN *NEUROSPORA CRASSA*

by

© M.S. ISLAM

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE

STUDIES IN PARTIAL FULFILLMENT OF THE

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Sex hormones in Neurospora crassa", submitted by M.S. Islam, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Date November 22, 1969

Abstract

'Sex hormones' (i.e., sex and fertility inducing substances) have been isolated from cultures of crosses between Em A and Em a as well as from the homokaryons of Em A and Em a of Neurospora crassa. The extracts were found to possess the following biological properties:

1) The extracts when applied to cultures of single strains of Em A or Em a induced an increase in the number of visible protoperithecia and protoperithecia-like bodies as initiated by strain Em A; no visible protoperithecia and protoperithecia-like bodies were noted when Em a was used as the tester strain.

2) The extracts when applied to crosses between sterile and wild type strains enhanced the fertility of some sterile strains of mating type A but not of a sterile strain of mating type a.

3) The extracts when applied locally to a fertile cross will give rise to a chemotactic response in terms of localized perithecia.

4) In one case the extract from a cross Em A x Em a was found to induce 'selfing' of an Em A strain thereby giving rise to A, a, bisexual (self-sterile) and sterile progenies.

Biochemical investigations using thin-layer chromatography indicated that two substances are present in the cross extract of Em A x Em a whereas each of the single strain extracts (Em A or Em a) contained only one biologically active substance. The substances yielded lipid-positive colour test with spray reagents. Ultraviolet, Infrared, Nuclear Magnetic Resonance, Mass-Spectra and Micro analysis of the extracts of single strains (Em A and Em a) characterized the sex-inducing

substances as open chained, unsaturated and possibly branched hydrocarbons with a molecular weight of 354-372 (mating type a) and 344-357 (mating type A).

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INTRODUCTION

Moreau and Moruzi (1931, 1932) were the first investigators who demonstrated the existence of 'sex-hormone(s)' in Neurospora with the aid of classical U-tube experiments. Although Dodge (1931) and Aronescu (1933) were unable to confirm these findings, Lindegren (1936) and later Moreau and Moreau (1937, 1938) put forward evidence in favour of their existence. After the initial investigation by Moreau and Moreau (1937, 1938) interest in the fertility and sex-inducing substances in Neurospora declined and it was not until the work of Barbesgaard and Wagner (1959) that a new impetus was given to this area of research. These latter authors investigated the biochemical basis of protoperithecia formation in Neurospora crassa and reported a relationship between melanin synthesis and protoperithecia development. Meanwhile, Ito (1956) reported on the effect of culture filtrate of one mating type on the other and also of the filtrate from a fertile mating on either of the individual mating types. The present study constitutes a preliminary assessment of fertility and sex-inducing substances as obtained from fertile crosses and single strains of N. crassa by means of biochemical extraction procedures. Since these substances were found to be active in enhancing the development of protoperithecia, protoperithecia-like bodies and perithecia when used in minute quantities on wild type crosses, it was decided to test their activity on known female and male sterile and semi-sterile mutants and on single strains of either mating type. A gross description of these effects has been given. The evidence for the presence of biologically active compounds in cross and single strain extracts of N. crassa encouraged a reconnaisance into their biochemical identification.

LITERATURE REVIEW

The activity of specific diffusible substances in the initiation and regulation of the sexual process in fungi was first suggested by Sachs and De Bary in the 1880's. However, during the next 40 years no confirmation of this concept became available. Burgeff in 1924 demonstrated cross-stimulation between compatible strains of Mucor mucedo growing on opposite sides of a celloidin membrane. This observation provided for the first time a strong indication for the existence of substances active in the regulation of sexual processes. In the succeeding quarter century, the presence and biological activities of similar substances have been proven in a number of sub-groups of Phycomycetes and Ascomycetes: (I) in Achlya: Raper, 1951; Barksdale, 1960; (II) in Mucor: Banbury, 1954; 1955; Plempel, 1957; Plempel and Baumitzer, 1958; (III) in Ascobolus: Bistis, 1956; 1957; (IV) in Allomyces: Machlis, 1958a; 1958b; (V) in Saccharomyces: Levi, 1956; and (VI) in Bombardia: Zickler; 1952.

Sexual hormones may be defined as those substances "secreted by the individuals of the same or of closely related species as that in which their highly specific activities are elicited" ... (Raper 1960, p. 800). Secretions of a more general nature such as vitamins, amino-acids, purines and other growth accessories that frequently exert a marked effect upon sexual productivity are not included in the group of sexual hormones.

Raper states that ..."Coordinating mechanisms operating between the individuals by means of sexual hormones might be expected

on rational grounds to have evolved in those groups of organisms that inhabit an essentially aquatic environment and lack highly specialized, hence insulating, structural forms. Under these circumstances, distinct selective advantage would accrue to a species the individuals of which could interact sexually to diffusing metabolic products. Coupled with sexual or incompatibility differentiation, interaction dependent upon diffusing agents limits sexual activity to compatible individuals in close proximity, hence with the best possible prospects for the culmination of sexual process. In the forms having more highly elaborated structure, vegetative and sexual activities are not so nearly mutually exclusive and early stages in the sexual progression are internally regulated either without coordination between the individuals or coordinated by common external stimuli, e.g., photoperiod. Mechanisms regulating the entire sexual progression by means of secreted hormones thus appear likely and have been found among plants only in the Algae and Fungi" ... (Raper, 1960, p. 800).

That the initiation and coordination of the stages in sexual progression are controlled by hormonal mechanisms, has been demonstrated in considerable detail in a number of fungi. Good evidence has been found in Achlya, an aquatic biflagellate Phycomycete (Raper, 1951; Barksdale, 1960), Mucor, a Zygomycete (Burgeff, 1924; Krafczyk, 1935; Banbury, 1954; 1955; Plempel, 1957; Plempel and Braunitzer, 1958) and in the Discomycete, Ascobolus (Bistis, 1956, 1957; Raper, 1957). In addition to these cases there are a few reports of hormonal activities in certain single stages of sexual development in yet other groups of fungi, such as Allomyces, (Machlis, 1958a, 1958b) in Saccharomyces, an

an Ascomycete (Levi, 1956), and in Bombardia and probably Neurospora of the Pyrenomycetes (Zickler, 1952; Backus, 1939).

In recent studies (Levi, 1956; Brock, 1958a, 1958b, 1959) relating to the phenomenon associated with interclonal copulation of haploid yeast, a controlling mechanism for sexual interaction of different strains has been suggested. Levi (1956) has reported that for haploid strains of Saccharomyces cerevisiae the induction of copulating hyphal processes occur only amongst a (-) cells when mated with α (+) cells, grown on malt agar. The inducing agent was demonstrated as a diffusible one. Hyphal processes were developed on a cells when placed on agar following the removal of copulating a and α cells from the plate. Similarly, hyphal processes were induced amongst a cells when separated from α cells by a cellophane membrane. The diffusible agent, the de novo inducation of the copulatory processes and the specificity of reaction, all seem to be indicative of the existence of hormonal regulatory mechanisms.

Brock (1958a, 1958b, 1959) suggested a biochemical basis for agglutination of (+) and (-) cells of heterothallic yeast Hansenula wingei. He postulated that a protein is present on the surface of cells of one strain and a polysaccharide on the surface of the cells of the compatible strain. Agglutination of these two substances is presumed to be a prerequisite for sexual fusion. It was envisaged that agglutination depends on a complementary interaction of the two essential substances somewhat similar to the mechanism of an antibody - antigen reaction. However, in a recent report the two substances have been identified as glycoproteins (Brock and Crandall, 1968).

In addition, it was found that these substances were situated on the surface of the cells of the respective mating types. Surface-borne glycoproteins have also been reported in two species of Chlamydomonas (Forster and Wiese, 1954, 1955; Foster et al., 1956). These glycoproteins are essential in the clumping of sexually compatible gametes.

That the diffusible agents are capable of restoring normal sexual function has been demonstrated in Glomerella by Markert (1949) and by Driver and Wheeler (1955). Markert (1949) reported that diffusing agents from a "weakly-self-fertile" strain are able to restore normal fertility in a mating between two "weakly-interfertile strains." Driver and Wheeler (1955) reported that the addition of filtrate of a normal strain increased the frequency of fertile perithecia in a defective strain from about 2% to as much as 97% when compared with non-treated wild-type crosses.

In Neurospora, the presence and activity of 'sexual hormone(s)' has been a subject of much controversy. Their existence has been demonstrated by some investigators, while others were unable to corroborate these results. In 1931 Moreau and Moruzi claimed to have demonstrated 'sexual hormones' in Monilia, nowadays know under the name Neurospora. A U-tube filled with medium was inoculated at one end with strain M (isolated from a decaying mushroom) whereas the other end was inoculated with the original Dodge strain N. Although single strain cultures produced only small protoperithecia, in the U-tube culture the arm containing the strain N showed the development of large size perithecia (sclerotia) while in the arm containing strain M normal mature perithecia (sclerotia) were observed. Small portions of the agar content of the horizontal section of the U-tube were cut

out and incubated under humid conditions. No growth was observed; therefore, it was concluded that no hyphal growth between the two vertical ends of the tube had occurred. In the view of these authors, (loc. cit.) the production of perithecia was due to the action of diffusible hormones passing from one mycelium to the other through the solidified medium. However, in the same year Dodge (1931) repeated the U-tube experiments, using one conidial strain and one non-conidial strain. Perithecia appeared in the ends of the tubes only when air spaces were formed by the shrinking agar medium and, therefore, were present throughout the greater portion of the length of the horizontal part of the U-tube. In cases where perithecia were initiated it was shown that both the strains, conidial and non-conidial, were present in the medium in the immediate vicinity of the perithecia.

In a subsequent paper Moreau and Moruzi (1932) claimed to have provided further evidence for a distant hormonal action in the induction of perithecia. Their new observations pointed to the presence of a wide "restraint area" in the region where compatible mycelia intermingle, together with the occurence of perithecia on either side of this area. Similar observations were made twenty years earlier by Dodge (1912) in Ascobolus.

In the following two years two papers by Aronescu (1933, 1934) subjected the hormone theory to intensive genetic analysis. Using compatible conidial and non-conidial strains and the U-tube technique, asci were isolated from perithecia whenever they appeared, and the sexual and conidial characteristics of the mycelia derived from the eight separated spores of each were determined. Theoretically,

three possibilities exist: a) segregation due to syngamy of both factors in a perfectly normal Mendelian ratio; b) no segration for both factors, all spores alike and identical to the parent strain, ('selfing' of a single strain under hormonal influence of the other); c) segregation for the sex factor, but conidial character always that of the 'sole' parent). In the analysis of about 50 asci the first situation was obtained for each ascus, proving conclusively that the distance between the two ends of the tube has been bridged by hyphal growth and that compatible nuclei has been brought together. The same results were again obtained when the work was repeated using the same strains as used previously by Moreau. Aronescu also disputed Moreaus' further claim that two different mycelia (but of same mating type) when grown together on a single plate, produce perithecia. In many trials her results were uniformly negative (see Raper, 1952, p. 526).

Lindegren (1936) claimed to have confirmed the existence of sex hormone(s) in Neurospora. A bisexual, heterokaryotic, self-sterile strain (containing f+ and f- nuclei which were incapable of copulation because of sterility factors), was mated to a highly fertile mutant strain (F+). Two kinds of zygotes resulted: (1) f+/f- and F+/f-. According to Lindegren hormones from the F+ strain had enabled the sterile strains (f+ and f-) to copulate. Although in their initial investigation, Moreau and Moruzi (1931), assumed that their wild type Neurospora strains were homokaryotic and that hormones had stimulated them to reproduce parthenogentically, in the light of Lindegren's work their strains were probably bisexual and self-sterile, and hormones possible supplied an essential substance for fruiting.

Commenting on the work of Dodge and Aronescu, Lindegren (1936) states that: "in Dodge and Aronescu's experiments homokaryotic (+) and (-) strains were used in two arms of the U-tube. Such an experiment cannot test the production of hormones. They also used single conidium cultures of the Moreau and Moruzi wild strain in an attempt to reproduce Moreau and Moruzi experiments." According to Lindegren (loc. cit.) such an experiment is "particularly liable to fail because by making single conidium isolates, the heterokaryon is easily separated". (Lindegren, 1936, p. 405).

In 1937 Moreau and Moreau described experiments in which they introduced further evidence in favour of the existence of sex-hormones in Neurospora. In a cross of N. sitophila with N. tetrasperma some asci were detected which contained spores of the tetrasperma type. It was, therefore, concluded that the origin of these spores stemmed from the 'selfing' of tetrasperma type nuclei under the influence of 'sex-hormones'.

A final paper by Moreau and Moreau (1938) cited new but convincing 'evidence' for hormonal activity in Neurospora. Mycelia of one sexual strain, growing on an agar plate, were heated for ten minutes in steam at atmospheric pressure to kill the vegetative thallus. One centimeter square blocks of agar were cut out from this plate and placed on the surface of a living mycelium of a strain of opposite mating type. It was observed that perithecia developed on this compatible strain. A negative result was obtained if the temperature was raised to 110°C. At a killing temperature of 90°C-94°C perithecia were formed in every case. Later Moreau (loc. cit.) treated the

mycelium by ether or chloroform vapour instead of heat-killing and obtained similar results. In their conclusion they stated that the results indicate the presence of hormones in the medium of the killed thallus which subsequently on transfer initiated perithecia in the compatible strain. However, no genetical analysis of the sex and conidial characters of the progeny from 'induced' perithecia was carried out.

Backus (1939), while studying the mechanics of ascogonial fertilization in Neurospora sitophila, observed two phenomena which indicated hormonal activities. It was found, for instance, that the germination of conidia was completely inhibited in regions previously overgrown by mycelium of the opposite compatibility strain. This effect was interpreted as being due to an inhibiting substance, provided by the opposite mating type. In addition, it was observed that the long sparsely branched trichogyne did not produce short lateral branches in the absence of conidia, micro-conidia or hyphal fragments of the opposite compatability strain in the immediate vicinity. In the presence of potential fertilizing elements, one to several lateral branches were regularly observed to arise upon the trichogyne and grow directly towards the source of the fertilizing nuclei.

Hirsch (1954), and Barbesgaard (1959), nearly two decades after the publication of Backus (loc. cit.), provided some information with regards to environmental and biochemical factors involved in protoperithecia formation and postulated that the incidence of proto-perithecia development is directly proportional to the melanin content of the fungus.

Meanwhile, Ito (1956) reported the effect of culture filtrate on the induction of protoperithecia formation in Neurospora. He found that the filtrate of medium containing mycelia of a single strain of either mating type was less effective in stimulating perithecia formation than the filtrate of medium containing mycelia of both mating types. The effect of mixed culture filtrate was found to surpass the additive effect of two single culture filtrates of each mating type. This observation was taken to indicate the presence of a cooperative but unknown second factor in the mixed culture filtrate. In fact, the single culture filtrate of one mating type stimulated the formation of (proto) perithecia on mycelia of opposite mating type; although these (proto) perithecia failed to produce asci and ascospores. Ito (loc. cit.) concluded that the formation of perithecia on the haploid vegetative thallus of one mating type was stimulated by a diffusible substance secreted by mycelia of the opposite mating type, quite irrespective of sexual fusion of both mating types.

Finally, Ahmad et. al., (1967) reported that filter sterilized cross mycelial extract increased the fertility of semi-sterile mutants of the lys-5 locus. Mycelial extracts of single strain cultures (either Em a or leu-1 A) as well as culture filtrates (Em a, leu-1 A and Em a x leu-1 A) did not have a positive effect on the restoration of the weakly-fertile mutants of the lys-5 locus.

MATERIALS AND METHODS

1. Strains:

All of the strains of Neurospora crassa used in the present study were obtained from the Fungal Genetics Stock Centre, Dartmouth College, New Hampshire, except for the sterility mutants (5366-A, 7232-A, 10710-A, 9312-A, 10402-A and 8455-a) which were obtained from Dr. N. Vigfusson. Descriptions of the strains employed are given in Table I.

2. Media Used:

(a) Minimal medium (Vogel, 1956): Vogel's medium (N), when necessary supplemented with 100 mg/1000 ml. of required amino acids, was used throughout the investigation.

(b) Crossing medium: The crossing medium was basically that of Westergaard and Mitchell (1947) with slight modifications as outlined in the Stanford Neurospora Methods (1963). Biochemical requirements were added when necessary (100mg/1000 ml. of medium).

Table - I

DESCRIPTION OF THE STRAINS OF NEUROSPORA CRASSA USED

(a) Stock Cultures.

Stock No.	Marker	Allele No.	Linkage Group	Mating Type	Remarks
FGSC 691	Emerson-A	-	IV R	A	
FGSC 692	Emerson-a	-	IV R	a	
FGSC 45	fl	Lindegren (L)	II R	A	Emerson background
FGSC 732	hist-2	K 271	I R	a	Emerson background
FGSC 668	hist-2	Y 153M43	I R	A	St. Lawrence backgroun
FGSC 99	al-2	15300	I R	A	Mixed background

(b) Sterility mutants (obtained from Dr. N. Vigfusson).

Group	Isolate No.	Biochemical Marker	Mating Type	Description	Remarks
1	5366	pan-1	A	Completely sterile, producing only abundant very small brown proto-perithecia. (1)	Male and female sterile. St. Lawrence back-ground, UV induce
2	7232 10710	leu-3 leu-3	A) A)	Abundant small brown protoperithecia with occasional mature perithecia, (2) abundant normal ascospores.	Male and female sterile. St. Lawrence back-ground, UV induced.
3	9312	leu-3	A	Abundant immature brown perithecia, ostiole absent, no spores.	Male and female sterile (as a female parent it produces a few perithecia with spores). St. Lawrence back-ground, UV induce

Table - I (Continued)

Group	Isolate No.	Biochemical Marker	Mating Type	Description	Remarks
4	10402	leu-3	A	Abundant normally pigmented but immature perithecia; perithecia empty or containing few spores.	Male and female sterile (semi-sterile). St. Lawrence background, UV induced.
5	8455	wild	a	Produces some perithecia with spores (semi-sterile) when used as male parent.	Male sterile. St. Lawrence background, UV induced.

1) Although protoperithecia are defined as the female sex organ (unfertilized) surrounded by a thick web of "mantle" hyphae together with numerous radiating hyphae, the present author necessarily had to include protoperithecia-like bodies within this classification which in a mature cross do not develop into perithecia and hence may be devoid of an ascogonium. Protoperithecia initials not visible under 2X magnification were not taken into account.

2) Perithecia defined as fully developed fruiting bodies after karyogamy.

3. Methods:

I. Culture of strains:

(a) Culture of crosses: For the extraction of sex and fertility inducing substances from an established cross between wild type strains with an Emerson genetic background, 15 ml. of sterilized liquid crossing medium was poured in a single petri dish (size 100 x 15 mm.) in which 5 sheets of sterilized filter paper (#1, Ø 9 cm.) were placed for the support of mycelial growth. The 15 ml. of liquid crossing medium was entirely absorbed by the amount of filter paper in each petri dish. Inocula of both Em A and Em a were made simultaneously on the filter paper close to one another and the culture was incubated at a temperature of 23° C to 26° C for three weeks, by which time free ascospores could be detected. In some experiments the cultures were allowed to grow only for two weeks. Usually, 50 plates were grown in a single experiment.

(b) Single strain culture: In single strain cultures, the same procedures as mentioned under the heading 'culture of crosses' were employed. Minimal medium was used for culturing single strains. (see page 11).

II. Biochemical Procedures:

(a) Water extraction: Extraction was made after two or three weeks of incubation of mycelia. The filter papers (50 x 5) with the adhering mycelia were soaked in 3 litres of sterilized distilled water for about two hours under occasional stirring with a glass rod. Subsequently, the water was decanted and the filter papers with the mycelia still adhering were soaked again in an

additional 3 litres of sterilized distilled water. In total, 6 litres of water-extract were obtained in this manner. The water-extract was then filtered through a filtering apparatus composed of 8 layers of cheese cloth together with a thick glass wool-pad and finally 3 layers of Whatman filter paper #1.

(b) Charcoal extraction: Five grams of activated charcoal (Norit-A) was added to 500 ml. of the sterilized water extract (see page 14). The mixture was agitated for a few minutes after which it was kept at room temperature for about 3 hours. By this time the charcoal in the mixture had sedimented at the bottom of the flask. Subsequently, as much water as possible was decanted without disturbing the charcoal sediment. The remaining water together with the charcoal was then filtered through Whatman filter paper #1 and the charcoal so collected was dried at room temperature.

(c) Solvent extraction:

i. <u>Extraction with chloroform</u>

Three hundred ml. of chloroform (reagent grade) was added to the dried charcoal (see above) and agitated vigorously for a few minutes. The mixture was kept at room temperature for about three hours under occasional and mild agitation, after which the chloroform mixture was filtered twice through 5 layers of Whatman filter paper #1 ensuring a complete separation of the two components. This extraction procedure was repeated once more and finally about 500 ml. of chloroform extract was obtained in this manner. The extract was then evaporated to dryness under vacuum suction. The residue, consisting of a thin film of an oily substance was redissolved

in 15 ml. of chloroform, collected in a specimen vial and kept under refrigeration.

ii. Extraction with Chloroform - Methanol Solution

Three hundred ml. of 2:1 chloroform - methanol solution (Folch et al., 1957) was added to the charcoal obtained from a water extraction procedure and kept at room temperature for three to several hours under occasional agitation after which the solvent was recovered by filtering the mixture twice through 5 layers of Whatman filter paper #1. This extraction and filtration procedure was repeated and a total of about 500 ml. of extract was obtained in this manner. Distilled water (0.2 by volume) was added to the extract after which the mixture was allowed to separate into two layers. The bottom layer (chloroform-methanol layer) was collected and evaporated under vacuum (suction) to dryness. The thin film of the resulting oily residue was redissolved in 15 ml. of chloroform and kept under refrigeration. The top layer (water) was discarded.

iii. Extraction with Acetone

The procedure for acetone extraction was basically the same as the one used for the extraction with chloroform except for the fact that acetone was used instead of chloroform. The final residue after evaporation of acetone consisted of an oily film which was redissolved in acetone and stored under refrigeration.

iv. Extraction with Ethyl Alcohol (95%)

The alcohol extraction procedure was the same as mentioned for chloroform except that 95% alcohol was used as the

solvent. In all cases, the alcohol extract on final evaporation did not yield a visible residue.

On preliminary testing of the different extracts on wild type crosses it was found that the chloroform, the chloroform-methanol and acetone extracts showed biological activity in enhancing the number of perithecia. The alcohol extract showed no effect. The chloroform extraction procedure was adopted as the standard extraction method for later experiments.

(d) Purification of the extracts:

For the purification of Neurospora extracts (chloroform and chloroform-methanol extracts) the method followed was basically that of Folch et al., (1957) for lipids. Re-distilled water (0.2 by volume) was added to the final extract, mixed thoroughly and the mixture was allowed to separate into two layers for 15 to 30 minutes after which the upper layer (water) was siphoned off carefully and discarded. The process was repeated once more, after which the solvent layer was stored under refrigeration.

III. Biological Characterizations:

(a) Preparation of culture plates: Westergaards's standard crossing medium was used throughout the preparation of crossing plates. Initially, 15 ml. of sterilized crossing medium was poured in each petri plates (100 x 15 mm.). In later experiments, however, 20 ml. of the medium was used per plate to prevent drying out of the plates during the incubation period.

(b) Addition of extract:

The amount of extract supplemented to each plate was 0.1 ml., unless stated otherwise. The extract (0.1 ml.) was either (1) dried on a sterilized triangular piece of filter paper which was then placed at the center of the agar plate, or (2) pipetted directly onto the plate

Preliminary tests using an Em A x Em a extract (0.1 ml.) in the amount of 2 µg/plate produced an enhancement of protoperithecia and protoperithecia-like bodies development in an Emerson A culture. The same quantity of extract produced a three-fold increase in the number of perithecia produced by the cross Em A x Em a.

Filter-paper triangles were made by cutting a filter paper (#1, Ø 5.5 cm.) into four equal parts.

The control consisted of a test for possible biological activity of chloroform on the development of protoperithecia and protoperithecia-like bodies. For the preparation of control plates, 500 ml. of chloroform was evaporated to 15 ml. and from this volume 0.1 ml. was added either directly to each of the control plates, or onto triangular filter paper pieces placed at the center. In each case the extract was checked for possible bacterial or fungal contaminants by plating 0.1 ml. of extract/plate.

(c) Preparation of Neurospora crosses:

i. Cross of wild types (A x a) and A (sterile) x a (wild)

The crossing plates were prepared with 20 ml. of sterilized Westergaard's crossing medium. Each crossing plate was inoculated at two diagonally opposing locations, close to the circumference

of the petri dish, one location for the inoculum of mating type A and the other for a. Each experiment was replicated at least 5 times. The crosses were allowed to grow at room temperature at 23° C to 26° C for three weeks. Observations were made at 7-day intervals. Photographic documentation of the experiment was carried out after 21 days of incubation.

ii. Cross of fluffy - A x a (male sterile)

The morphological mutant fluffy - A was grown as a protoperithecial strain. Five replica plates each with extract added (at the center of the plate) were incubated for 72 hours at 23° C to 26° C together with 5 control plates (without extract), after which all plates were spermatized with 0.5 ml. of conidial suspension of the male sterile strain 8455-a (conidial density 2.6 x 10^6 per ml.). Observations were made weekly. Photographic documentation was carried out after 21 days of incubation.

(d) Preparation of Neurospora Single Strain Cultures:

Crossing plates were prepared with 20 ml. of sterilized Westergaard's crossing medium. Each crossing plate was inoculated close to the circumference of the petri dish with either an Em A or an Em a wild type strain. Each experiment was repeated at least 3 times (usually 5 times). The single strain cultures were allowed to grow at 23° C to 26° C for three weeks. Observations were made at 7-day intervals. Only visible (magnification 2X) protoperithecia and protoperithecia-like bodies were counted.

IIIA. Isolation of the hist-2 A, mutant with Emerson background:

Em A was grown as protoperithecial strain. After 72 hours of incubation the plates were spermatized with conidia from hist-2 a (Emerson background) mutant after which the plates were re-incubated at a temperature of 23° C to 26° C for approximately three weeks until 'spore shed' could be observed. Seventy-five single ascospores were isolated and after a heat shock at 60° C for 30 minutes these ascospores were allowed to germinate on a histidine-supplemented minimal agar medium. After selection of histidine-requiring segregants, mating type tests were performed on these isolates by crossing them to both Em A and Em a. Only one of the hist- isolates was found to be of mating type A.

BIOCHEMICAL IDENTIFICATION:

(a) Thin-layer chromatography

Thin-layer chromatography was carried out with an Eastman chromatogram developing apparatus. This apparatus consists of a sandwich-type chamber with a small internal volume enabling fast saturation with solvent vapour. A consistency of results was obtained with this apparatus throughout experimentation. Either full 20 x 20 cm. sheets or cut strips of silica gel were affixed between the plates, supported in such a manner that the eluant was able to travel by means of surface tension. In addition a conventional thin-layer chromatography tank was employed. Due to the resulting inadequate separation this method was abandoned at an early stage of experimentation. The determination of suitable solvent systems was initially carried out in wide-mouth bottles of about 15 cm. height (vol: 500-600 cc.) using silica gel strips.

i. Chromatography Solvents

To determine the best solvent system for the separation of the compounds the following solvents were tried:

1. Chloroform/Benzene: (3:1, 4:1, 6:1 v/v)
2. Benzene
3. Hexane
4. Carbon Tetrachloride
5. Propanol/Ammonia (25%) (2:1 v/v)
6. Propanol/Water (7:3 v/v)
7. Butanol/Acetic Acid/Water (80:20:20 v/v)
8. Hexane/Ethyl ether/Acetic Acid (80:20:1 v/v)
9. Hexane/Ethyl ether (9:1 v/v)

ii. UV Fluorescence

UV fluorescence of the developed chromatograms was observed under a CAMAG universal UV lamp at wave length of 2540Å and fluorescing spots were marked accordingly with a lead pencil.

iii. Chromatography

Eastman chromatography sheets (20 x 20 cm.) of silica gel (6060) with fluorescence indicators were used for thin-layer chromatography. The characteristic of this sheet is that the silica gel is located on the plastic sheet and in addition, a fluorescence indicator (lead-manganese activated calcium silicate) is present which renders non-fluorescing substances to fluoresce at 2540Å.

iv. Spray - Reagents

For the detection and determination of the developed fluorescing spots the following spray-reagents were tried:

1. Iodine crystals - (Test for lipids)
2. 1% Phosphomolybdic acid in ethyl alcohol - (Test for lipid-protein complexes)
3. 0.4% solution of Bromothymol blue in 0.01 molar sodium hydroxide - (Test for lipids)
4. 2',7'-Dichlorofluorescein solution (0.2% solution) in ethyl alcohol - (Test for fatty esters)
5. Ninhydrin Solution: 0.3% ninhydrin in butyl alcohol containing 3% glacial acetic acid - (Test for amino acids).

Visualization 1. The chromatogram strips were placed in a jar containing iodine vapour for about five minutes. In the presence of lipids the fluorescing spots develop a brown colour within one minute.

Visualization 2. The chromatogram strips were sprayed with, or dipped into the solution of phosphomolybdic acid solution and heated for 15 minutes at 100^{o} C. A blue colour originates in the presence of lipids.

Visualization 3. A spray with Bromothymol blue solution renders the UV fluorescing lipid spots yellow.

Visualization 4. The spray reagent 2',7'-Dichlorofluorescein yielded no visible spots (either in bright light or under UV light) indicating the absence of fatty esters.

Visualization 5. Ninhydrin solution was sprayed on the chromatograms and heated gently for about 10 minutes. Since no spots developed it was concluded that amino-acids were absent.

v. Spot application method

Six pencil marks were made at equal distances about 2 cm. away from the bottom of the silica gel sheet. The extract was delivered by means of 5 µl. chromatography pipette at the indicated pencil marks taking care that the diameter of the spot did not extend beyond half a centimeter. Initially, different volumes (10 µl, 15 µl, 20 µl, 25 µl and 30 µl) of extract were delivered but in every case the same number of fluorescing spots were recovered in the resulting chromatograms. Routinely, therefore, chromatograms were prepared using 20 µl of extract. Control chromatograms were also run along with the extract and to these only the solvent was delivered (in the appropriate quantity).

(b) UV, Infrared, Mass and NMR Spectroscopy

i. Ultraviolet Spectroscopy

Ultraviolet spectra (UV) were determined with a UV spectrometer: model Jasco/UVs.

ii. Infrared Spectroscopy

Infrared (IR) spectra were determined with a Perkin Elmer-421 IR Spectrometer.

iii. Mass Spectroscopy

Mass-Spectra were recorded on a 70 eV MS:9 Mass-Spectrometer apparatus manufactured by Associated Electrical Industries Ltd.

iv. Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR Spectra were recorded on a Varian HA-100 instrument in deuterio-chloroform ($CDCL_3$) solution.

(c) Carbon-hydrogen determination

A Coleman C-H Analyser (model-33) was employed for the determination of the C/H ratio.

(d) Molecular Weight determination

An Osmometer (Mercro Lab., model-301A) was used for the molecular weight determination by means of the differential vapour pressure method.

EXPERIMENTS AND RESULTS

CHARACTERIZATION OF THE EXTRACT OF CROSS Em A X Em a

(a) Biological Characterization

i. The effect of the extract of cross Em A x Em a on wild type strains

The extract of the cross Em A x Em a was tested for the enhancement of protoperithecia development of the wild type strains Em A and Em a. On visual examination of the treated plates it was concluded that the extract of the cross Em A x Em a increased the number of protoperithecia developed by the wild type strain Em A, but no increase in the number of protoperithecia was noted when Em a was used as a tester strain.

Many small protoperithecia with a few large, well developed protoperithecia were initiated in the culture plates of tester strain Em A and treated with the extract whereas Em a cultures showed no reaction. The control plates in both cases were devoid of any visible protoperithecia except for Em A plates which occasionally produced a few (2-5) protoperithecia and protoperithecia-like bodies after three weeks incubation (Table II, Plate I - III). The experiment was also repeated with different concentrations of the extract (0.2, 0.3, 0.4, 0.5 ml/plate). Using Em A as a tester strain, it was observed that an increase in the concentration of the extract yielded an increase in the number of protoperithecia and protoperithecia-like bodies per plate. In some instances, this experiment yielded fertile perithecia with mature spore content (see Appendix). It was noted however, that an increase of the concentration of the extract even after treatment with an extract concentration of 0.5 ml/plate did not

have any visible effect on the development of protoperithecia and protoperithecia-like bodies by tester strain Em a (Table III, Plates IV and V).

TABLE - II.

EFFECT OF THE EXTRACT OF CROSS Em A X Em a ON SINGLE WILD TYPE STRAIN CULTURES (Em A AND Em a)

Strain	Extract	Seven-day plates (Development of Proto-perithecia)			Fifteen-day plates (No. of Protoperithecia)					Twenty-one-day plates (No. of Protoperithecia)					Remarks
		1	2	3	1	2	3	M	$S_{\bar{x}}$	1	2	3	M	$S_{\bar{x}}$	
Em A	chloroform 17/6/68	+	+	+	45	50	40	45.0	5.00	45	50	43	46.0	3.60	A few proto-perithe were well develop
	chloroform 2/8/68	+	+	+	25	27	25	25.7	1.15	28	27	25	26.7	1.52	
	chloroform-methanol control	+	+	+	25	17	28	23.3	5.68	25	17	28	23.3	5.68	
	control	-	-	-	-	-	-	-	-	-	-	-	-	-	
Em a	chloroform 17/6/68	-	-	-	-	-	-	-	-	-	-	-	-	-	The extract showed to have no effe on Em a
	chloroform 2/8/68	-	-	-	-	-	-	-	-	-	-	-	-	-	
	chloroform-methanol control	-	-	-	-	-	-	-	-	-	-	-	-	-	

Note: +: Development of visible protoperithecia and protoperithecia-like bodies.

-: No development of visible protoperithecia.

Table - III.

DOSE EFFECT OF THE EXTRACT OF CROSS Em A X Em a ON SINGLE WILD TYPE STRAIN CULTURES (Em A AND Em a)

Strain	Concentration of Extract	7-day plates (Development of protoperithecia)				15-day Plates								
						No. of protoperithecia				Avg.	No. of developed protoperithecia			Avg.
		1	2	3	4	1	2	3	4		1	2	3	4	
Em A	0.1 ml.	+	+	+	+	35	38	32	38	35	3	5	3	5	4
	0.2 ml.	+	+	+	+	42	40	33	34	37	2	5	3	4	4
	0.3 ml.	+	+	+	+	45	40	42	38	41	5	3	7	4	5
	0.4 ml.	+	+	+	+	48	46	60	55	52	6	6	4	7	6
	0.5 ml.	+	+	+	+	68	65	52	54	60	12	10	8	10	10
	Control	-	-	-	-	-	-	-	-	0	-	-	-	-	0
Em a	0.1 ml.	-	-	-	-	-	-	-	-	0	-	-	-	-	0
	0.2 ml.	-	-	-	-	-	-	-	-	0	-	-	-	-	0
	0.3 ml.	-	-	-	-	-	-	-	-	0	-	-	-	-	0
	0.4 ml.	-	-	-	-	-	-	-	-	0	-	-	-	-	0
	0.5 ml.	-	-	-	-	-	-	-	-	0	-	-	-	-	0
	Control	-	-	-	-	-	-	-	-	0	-	-	-	-	0

Note. +: Development of visible protoperithecia and protoperithecia-like bodies

-: No development of visible protoperithecia

ii. The Effect of the Extract of Cross Em A x Em a on Sterile Isolate I-5

One of the sterile isolates from the 'selfed' progeny of Em A (see Appendix, Table IV) was subsequently used as a tester strain to determine as to whether or not the Em A x Em a extract had any influence on the fertility of this sterile isolate. The isolate #I-5 (perithecium #I, random spore #5) was crossed to Em A as well as to Em a. Ten crossing plates were treated with the extract of the cross Em A x Em a whereas an additional ten plates (without extract) were used as a control. At seven-day intervals the plates were examined for a possible effect of the extract. It was found that cross I-5 x Em a treated with the extract developed perithecia after two weeks of incubation. The control plates in both crosses (I-5 x Em a, I-5 x Em A) showed no development of perithecia (occasionally one or two ill-developed perithecia (protoperithecia?) were found in some plates). On examining the cross I-5 x Em A treated with the extract (Em A x Em a) no difference in the number of perithecia was observed when compared with the control plates. The perithecia in the cross I-5 x Em a treated with the extract, showed spore-shed after four weeks. In repeating the experiment (I-5 x Em a) similar results were obtained (Plates VI and VII). In addition, single strain mixtures (1:1) of Em a and Em A were tested for their combined activity which was found to be less than that of the cross extract Em A x Em a. Due to the fact that analytical data are not available; no attempt has been made to interpret that experiment.

iii. The Effect of the Extract of Cross Em A x Em a on Isolate #I-28

Isolate I-28 (from the selfing of Em A; see Appendix, Table IV) is fertile when crossed to either tester strain Em A or Em a. It is for this reason that this isolate can be classified as bisexual but self-sterile. As an isolate, it will produce visible protoperithecia (30-35 per plate). In employing I-28 as a tester strain for the biological effect of the extract of cross Em A x Em a, ten replica plates were treated with the extract whereas an additional ten plates (without extract) were kept as control. Observations with regard to the initiation of protoperithecia were made at weekly intervals. It was found that the extract had a suppressive effect on the development of protoperithecia by isolate I-28. Consequently more protoperithecia were observed in control plates than in the plates treated with the extracts (Table V, Plates VIII and IX). Seventy-five well-developed protoperithecia from the control plates together with 42 well-developed protoperithecia from the plates treated with the extract were dissected after six weeks of incubation. In both cases all protoperithecia were found to be void of ascospores.

TABLE V.

EFFECT OF THE EXTRACT OF Em A X Em a ON THE BISEXUAL ISOLATE #I-28

	PLATES												Remarks
	1	2	3	4	5	6	7	8	9	10	M	$S_{\bar{x}}$	
With Extract	24	18	21	25	28	28	24	17	19	26	23.0	4.02	The extract has a suppressive effect on the protoperithec initiation by Isolat #I-28. (1)
Control	35	35	30	38	38	33	40	24	30	30	33.3	4.87	

(1) Protoperithecia count after four weeks.

iv. The Effect of the Extract Em A x Em a on the Cross Em A x Em a

Chloroform extract, chloroform-menthol extract and acetone extract of the cross Em A x Em a were found to increase the number of perithecia developed by the cross Em A x Em a. A five to ten times increase in the number of perithecia produced by the cross Em A x Em a in the presence of different extracts was observed when compared with the controls. The addition of extract to the filter paper in the medium gave rise to a strong chemotactic response by the organism in that most of the perithecia developed on the treated area in a congregated manner. In the control plates in which the filter papers did not contain extract, the perithecia developed evenly over the entire plate (Plate XI).

v. The Effect of the Extract of the Cross Em A x Em a on Sterile Biochemical Mutants of Mating Type A (St. Lawrence Background

Sterile mutants 5366-A, 10710-A, 7232-A, 9312-A, and 10402-A were used as tester strains for determining the biological effect of the cross-extract (Em A x Em a) on protoperithecium development and restoration of fertility. Of these strains, 5366-A was a pantothenic acid deficient mutant whereas 7332-A, 10710-A, 9312-A, and 10402-A were leucine deficient mutants. The extract proved to have a positive biological effect on all biochemical mutants with respect to the enhancement of the number of protoperithecia developed by these strains. In addition, the extract improved the fertility of all the biochemical mutants except for the mutant 5366-A which remained completely sterile.

This latter mutant (5366-A) is characterized by complete male and female sterility, and under control conditions this mutant produces an abundance of small and brown coloured protoperithecia and protoperithecia-like bodies when crossed to Em a. On treatment with the extract, mutant 5366-A developed a large number of perithecium-like bodies when crossed to Em a. In the control plates, however, only very small brown protoperithecia were found together with an occasionally ill-developed perithecium. The perithecia(?) arising in the crosses between 5366-A x Em a when treated with the extract were found to be immature and frequently without an ostiole. On dissecting these fruiting bodies, no ascospores were observed (Plates XII and XIII). Although attempts were made to express the enhancement of protoperithecial and perithecial development in a numerical manner, this parameter was abandoned due to the fact that severe clustering of protoperithecia and perithecia rendered counting procedures impossible. It is for this reason that the data have been presented by means of photographic documentation.

Mutants 7232-A and 10710-A, when crossed to Em a produced an abundance of small brown protoperithecia and protoperithecia-like bodies together with occasional mature perithecia (0-10 per plate) which contained normal ascospores. Crosses treated with the extract of Em A x Em a displayed many visible protoperithecia and protoperithecia-like bodies (7232-A: 70-140; 10710A: 140-160) together with some perithecia on the filter paper segment of the petri dish where the extract was applied. In control plates, however, this area was almost devoid of any visible protoperithecia and perithecia (Plates XIV and XV). The mature perithecia from the plates treated with the extract were found to contain ascospores.

Mutant 9312-A when crossed to Em a produced many immature brown perithecia(?) with no ostiole and without ascospores. However, in crosses between 9312-A and Em a (in which 9312-A functioned as the female (protoperithecial) parent an additional few perithecia with spores were found. The extract (Em A x Em a) elicted a very prominent effect on the cross 9312-A x Em a. Many perithecia (500-1000-fold increase over the control) developed and reach maturity and a heavy spore-shed was noted after three weeks of incubation. In the control plates the development of perithecia under similar experimental conditions remained scanty (Plate XVI).

Mutant 10402-A when crossed to Em a produced an abundance of normally pigmented but immature perithecia, either empty or containing a few spores. In the presence of the extract (Em A x Em a) the cross 10402-A x Em a produced a five to seven fold increase in the number of perithecia over that produced in the control plates (without extract). A strong chemotactic response of the organism with regard to the location of the extract was noted. Perithecia from treated crosses were normal in size and shape and displayed a heavy and early spore-shed (after two weeks of incubation) (Plate XVII).

vi. The Effect of the Extract of Cross Em A x Em a on the Semi-sterile Strain 8455-a (St. Lawrence Background)

Strain 8455-a (male semi-sterile) when used as a tester strain is a semi-sterile strain producing a few perithecia with ascospores. Crosses between 8455-a and fluffy-A (Emerson background) treated with the extract of Em A x Em a showed no increase in the number of perithecia when compared with the control plates (untreated) raised under identical environmental conditions. Due to the fact that 8455-a is male sterile and hence female fertile, fluffy-A was used as a protoperithecial strain.

(b) Biochemical Identifications

i. Determination of Solvent Systems for the Thin-layer Chromatography

All the solvents, as listed in the section dealing with materials and methods, were tested for their application in thin-layer chromatography. It was found that the chloroform-benzene solutions (3:1, 4:1, or 6:1 v/v) yielded on thin-layer chromatography two spots both detectable under UV light. One of these spots (Spot A) moves with the solvent front and fluoresced blue (B), whereas the other spot (Spot B) fluoresced light brown (LB) and does not move with the solvent front. Spot B appeared very faint under UV light. When other solvent systems were used for thin-layer chromatography, either only one spot could be detected under UV illumination, or a complete absence of fluorescing spots was noted. Solvents such as hexane/ethyl ether/acetic acid and hexane/ethyl ether developed two spots on thin-layer chromatograms. The appearance of these spots was far from satisfactory due to an absence of sharpness and well-defined boundaries. Consequently, a 3:1 chloroform-benzene solvent mixture was chosen as the standard solvent for thin-layer chromatography (Table VI, Plate XVIII).

ii. Spray Reagents Test

The developed thin-layer chromatograms (developed in 3:1 chloroform-benzene solution) were sprayed with, or dipped into, the spray reagents as listed under section dealing with materials and methods. Positive results were obtained with iodine, bromothymol blue, which were taken to indicate that the substances (as present in the fluorescing spots) are of a lipid nature (Table VII).

The negative reaction with phospho-molybdic acid indicates the absence of lipo-protein complexes.

iii. Biological Test for Activity of Thin-layer Chromatogram Spots

The biological activity of single thin-layer chromatogram spots (from Em A x Em a extract) was confirmed by testing their effect on protoperithecia development (including development of protoperithecia-like bodies) of wild type single strains as well as perithecia development of wild type crosses (Em A x Em a).

The spots were cut out of the chromatograms and added as supplement to test plates (1:1). The control consisted of a supplement of fragments of unused silica gel sheets of equal size. In all experiments the thin-layer chromatogram spots showed biological activity in enhancing the development of protoperithecia and protoperithecia-like bodies in a single strain culture of Em A, and improvement of fertility of the wild type cross Em A x Em a. No effect was noted when Em a was used as a tester strain.

Table - VI. DETERMINATION OF THE SOLVENT SYSTEM FOR THE CROSS-EXTRACT (Em A x Em a) IN ONE-WAY ASCENDING THIN-LAYER CHROMATOGRAPHY

	Solvent	Composition v/v	Ascending/ Descending	Paper	No. of Spots Developed	UV, Fluo-resence	Rf.	Nature of Spot	Sepa-ration of spots	Remarks
1.	Benzene		Ascending	(Silicagel with fluorescene indicator, Eastman 6060)	1	LB	1	Trailing	-	
2.	Butanol/Acetic Acid/Water	80:20:20	Ascending	"	1	B	.7	Condensed	-	
3.	Chloroform/ Benzene	3:1, 4:1 : 6:1	Ascending	"	2	1B 1LB	.8 .5	Condensed Condensed	Yes	Good
4.	Carbon Tetra-chloride		Ascending	"	None					
5.	Hexane		Ascending	"	None					
6.	Hexane/Ethyl Ether/Acetic Acid	80:20:1	Ascending	"	2	1B 1LB	.8 .7	Trailing	Yes	
7.	Hexane/Ethyl Ether	9:1	Ascending	"	2	1B 1LB	.9 .6	Condensed Faint	Yes	
8.	Propanol/ Ammonia (25%)	2:1	Ascending	"	1	B	.8	Trailing	-	
9.	Propanol/Water	7:3	Ascending	"	1	DB	.7	Trailing	-	

LB = Light Brown DB = Dark Brown B = Blue

Table - VII

SPRAY-REAGENTS TEST

Name of the Reagent	Reaction	Colour of Spots
1. Iodine Vapour	Positive	Brown (for both spots)
2. Phospho-molybdic Acid	Negative	----------
3. Bromothymol blue	Positive	Yellow (for both spots)
4. 2', 7'-Dichloro-fluorescein	Negative	----------
5. Ninhydrin	Negative	----------

CHARACTERIZATION OF THE EXTRACT OF STRAIN Em A AND OF STRAIN Em a

(a) Biological Characterization of the Extracts

i. The Effect of the Single Strain Extracts (Em A or Em a) on Wild Type Strains (Emerson Background)

Em A and Em a were tested with both the extracts of Em A and Em a. When the extracts of Em A or Em a were applied to tester strain Em A an increase in the number of visible protoperithecia and protoperithecia-like bodies initiated by Em A was noted. Tester strain Em a on the other hand did not respond to either extract. It was evident that in comparing the results obtained with the extracts of Em A and of Em a on tester strain Em A the extract of Em a developed more protoperithecia and protoperithecia-like bodies when applied to tester strain Em A than extract of Em A (Table VIII, Plates XIX and XX). Although a few protoperithecia in the plates of Em A were well developed, on dissection of these fruiting bodies no ascospores could be detected.

ii. The Effect of Single Strain Extracts (Em A or Em a) on the Cross Em A x Em a

Both the extracts (Em A or Em a) were found to have a positive effect on the fertility of the cross Em A x Em a. A four to six fold increase in the number of perithecia (when compared with untreated control plates) was noted. In the treated plates the test organisms responded chemotactically to the extracts (Plates XXI and XXII).

TABLE - VIII. EFFECT OF THE EXTRACT OF STRAINS Em A AND Em a ON SINGLE STRAIN WILD TYPE CULTURES (Em A AND Em a)

Extract	Strain	7-day plates (Development of proto-perithecia)				15-day plates (No. of proto-perithecia)						21-day plates (No. of proto-perithecia)						21-day plates (No. of large well developed proto-perithecia per plate)						Remarks
Number of Plate →		1	2	3	4	1	2	3	4	M	$S_{\bar{x}}$	1	2	3	4	M	$S_{\bar{x}}$	1	2	3	4	M	$S_{\bar{x}}$	
Em A	Em A	+	+	+	+	15	10	10	12	11.75	2.36	18	15	10	14	14.25	3.30	3	3	1	1	2.00	1.15	The extracts have no effect on the initiation of proto-perithecia by strain Em a.
	Em a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Em a	Em A	+	+	+	+	30	28	25	30	28.25	2.36	40	35	32	35	35.50	3.32	5	5	2	5	4.25	1.50	
	Em a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Control	Em A	-	-	-	-	-	-	-	-	-	-	2	2	3	2	2.25	0.50	-	-	-	-	-	-	
	Em a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Note: +: Development of visible protoperithecia and protoperithecia-like bodies.

-: No development of visible protoperithecia.

iii. The Effect of Single Strain Extracts (Em A or Em a) on Sterile Strains of Mating Type A (St. Lawrence Background)

Mutants 5366-A, 7232-A, 10710-A, 9312-A and 10402-A were tested with the extract of Em A and of Em a for possible enhancement of the development of protoperithecia and protoperithecia-like bodies and restoration of fertility. In the presence of the extracts, 5366-A developed a large number of perithecia-like bodies when crossed to Em a. The perithecia were mostly ill-developed and of small size. On dissection no ascospores were found. The effect of the single strain extracts on mutants 7323-A, 10710-A, 9312-A and 10402-A when crossed to Em a consisted of a general increase in the production of protoperithecia and protoperithecia-like bodies as well as perithecia (but less than noted for mutant 5366-A) together with an improvement of fertility (Plates XXIII, XXIV, XXV, XXVI, XXVII, XXVIII and XXIX).

iv. The Effect of Single Strain Extracts (Em A or Em a) on the Sterile Strain 8455-a (St Lawrence Background)

Mutant 8455-a was tested with the extracts of Em A as well as of Em a. The extracts proved to have no positive effect (i.e. in terms of an increase in the number of protoperithecia and perithecia) on the cross fl-A x 8455-a (semi-sterile).

v. The Effect of Single Strain Extracts (Em A or Em a) on a Sterile Isolate (I-5) from the 'Selfed' Progeny of Em A

Both the extracts showed a positive effect on the cross I-5 (Sterile) x Em a. Protoperithecia and protoperithecia-like bodies were developed in the treated plates. On dissection of resulting perithecia, ascospores were detected. From the results obtained it appears that the extract of Em a is more potent (70-fold increase over control)

in its biological effect than the extract of Em A (22-fold increase over control, Plates XXX and XXXI). In the control plates occasional (1-2) perithecium-like bodies (without any ascospores) were observed (Plate XXXII).

(b) Biochemical Identifications

i. Thin-layer Chromatography

The developed chromatograms (in 3:1 chloroform-benzene solvent, Plate XXXIII) were either sprayed with or dipped into the spray reagents listed in the section dealing with materials and methods. Positive results were obtained with Iodine vapour and Bromothymol blue solution, indicating that the substances present in the chromatogram spots are of a lipid nature (Table IX). Both the extracts of Em A and Em a yielded one spot on thin-layer chromatography (Rf. 0.8-0.9) which fluoresced blue under UV light of 2540Å. In either case the chromatogram spot reacted similarly to spray reagent tests with Iodine vapour and Bromothymol blue solution.

ii. Ultra-Violet Spectrum

The extracts, when dissolved in methanol, yielded on UV spectrometry an absorption shoulder at 265 mμ (Plate XXXIV).

iii. Infrared Spectrum

The following results were obtained with Infrared spectrometry of the single strain extracts (Em A and Em a) (dissolved in chloroform):

C-H stretching frequency: $\lambda_{max}^{CHCl_3}$ 2910cm^{-1} and 2860 cm^{-1}
C-H frequency: 1450 cm^{-1}, no carbonyl absorption

(Plate XXXV).

iv. Nuclear Magnetic Resonance Spectrum

The Nuclear Magnetic Resonance Spectrum showed three line patterns at 0.86 ppm. (CH_3-C) and a singlet at 1.22 ppm. (-CH_2-). (Plate XXXVI).

v. Mass Spectrum

Although Mass Spectrograms of the single strain extracts of Em A and Em a were indicative for hydrocarbons, insufficient resolution was obtained and hence an exact mass number could not be determined.

vi. Analytical Data

C/H Ratio:

The following data were obtained with the Coleman C-H Analyser:

Substance	Sample No.	Percentage of Carbon	Hydrogen
	1	85.14	12.95
Em A	2	85.01	12.19
	3	86.02	12.04
Em a	1	85.01	12.19
	2	85.62	12.41

Molecular Weights:

The molecular weights as determined by an osmometer were found to be as follows:

Substance	Molecular Weight	Solvent
Em A	1 : 357	Benzene
	2 : 344	Benzene
Em a	1 : 372	Benzene
	2 : 354.52	Benzene

Table - IX.

SPRAY - REAGENT TEST

Name of the Reagent	Reaction	Colour of Spot
1. Iodine Vapour	Positive	Brown
2. Phosphomolybdic acid	Negative	---
3. Bromothymol blue	Positive	Yellow
4. Ninhydrin	Negative	---
5. 2',7'-Dichloroflurescein	Negative	---

Plates I - III. Photographs showing the effect of the extract of the cross Em A x Em a on single wild type strain cultures, Em A (Plate I) and Em a (Plate II) when compared with untreated control (Plate III).

PLATE I.

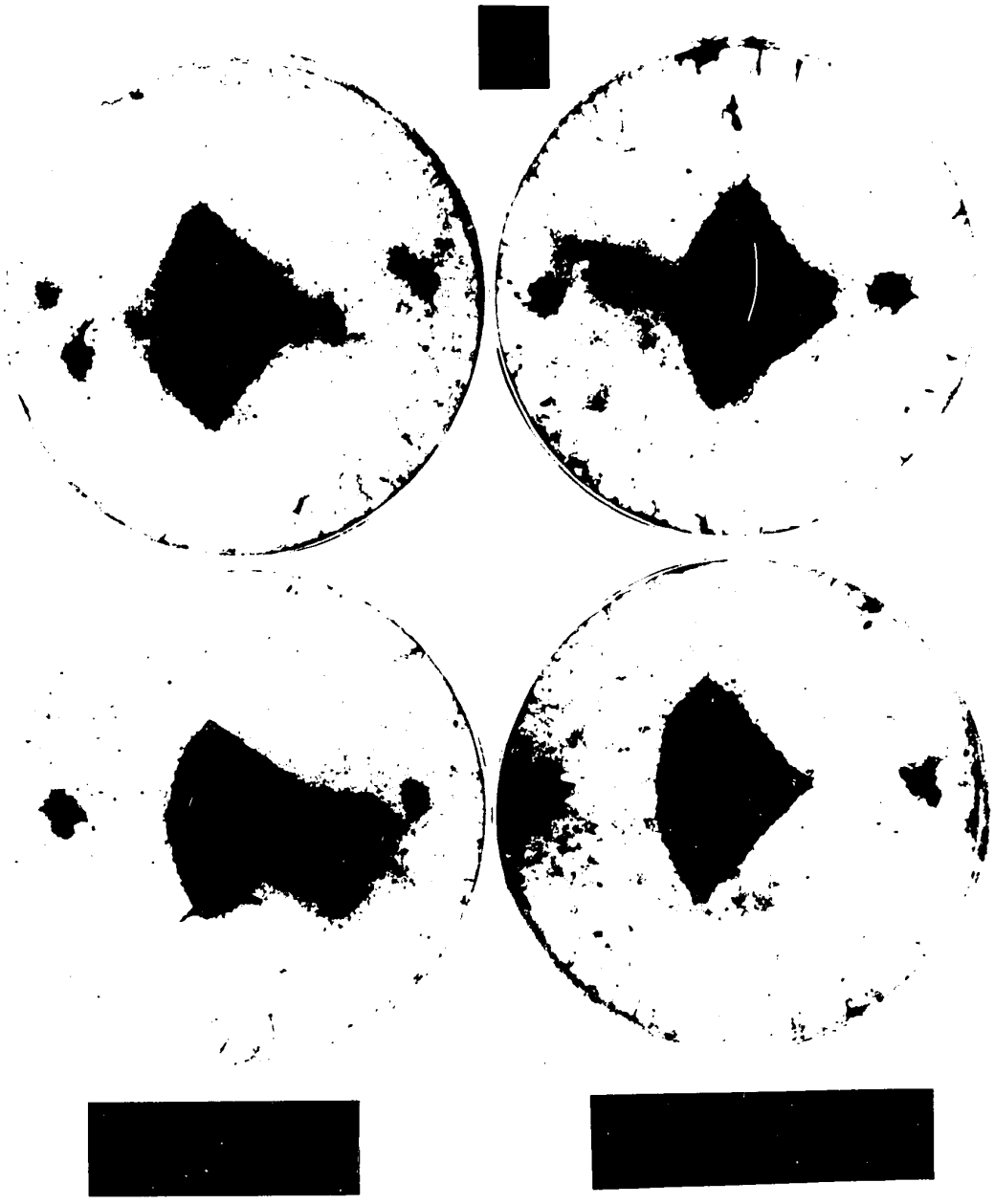


PLATE II.

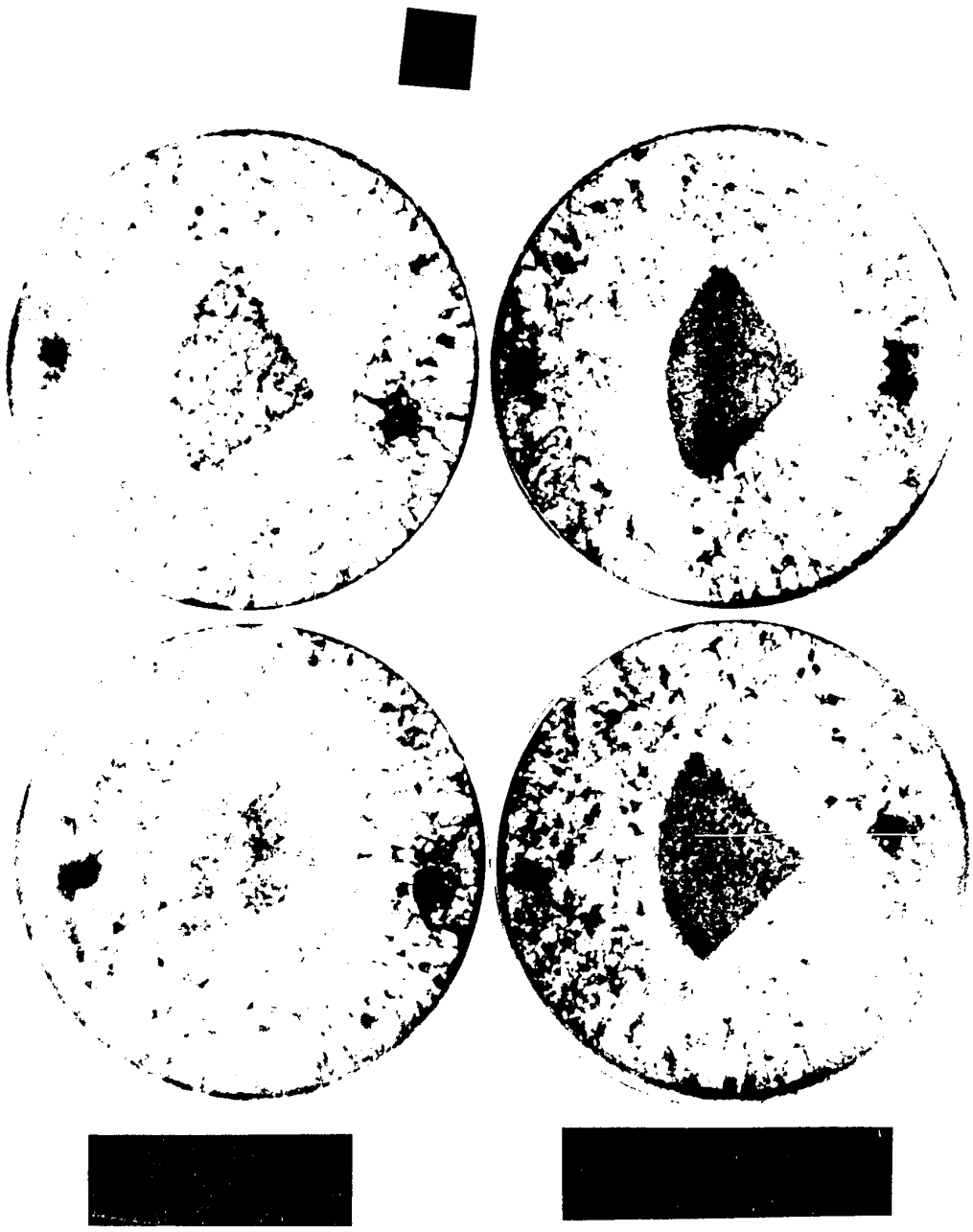
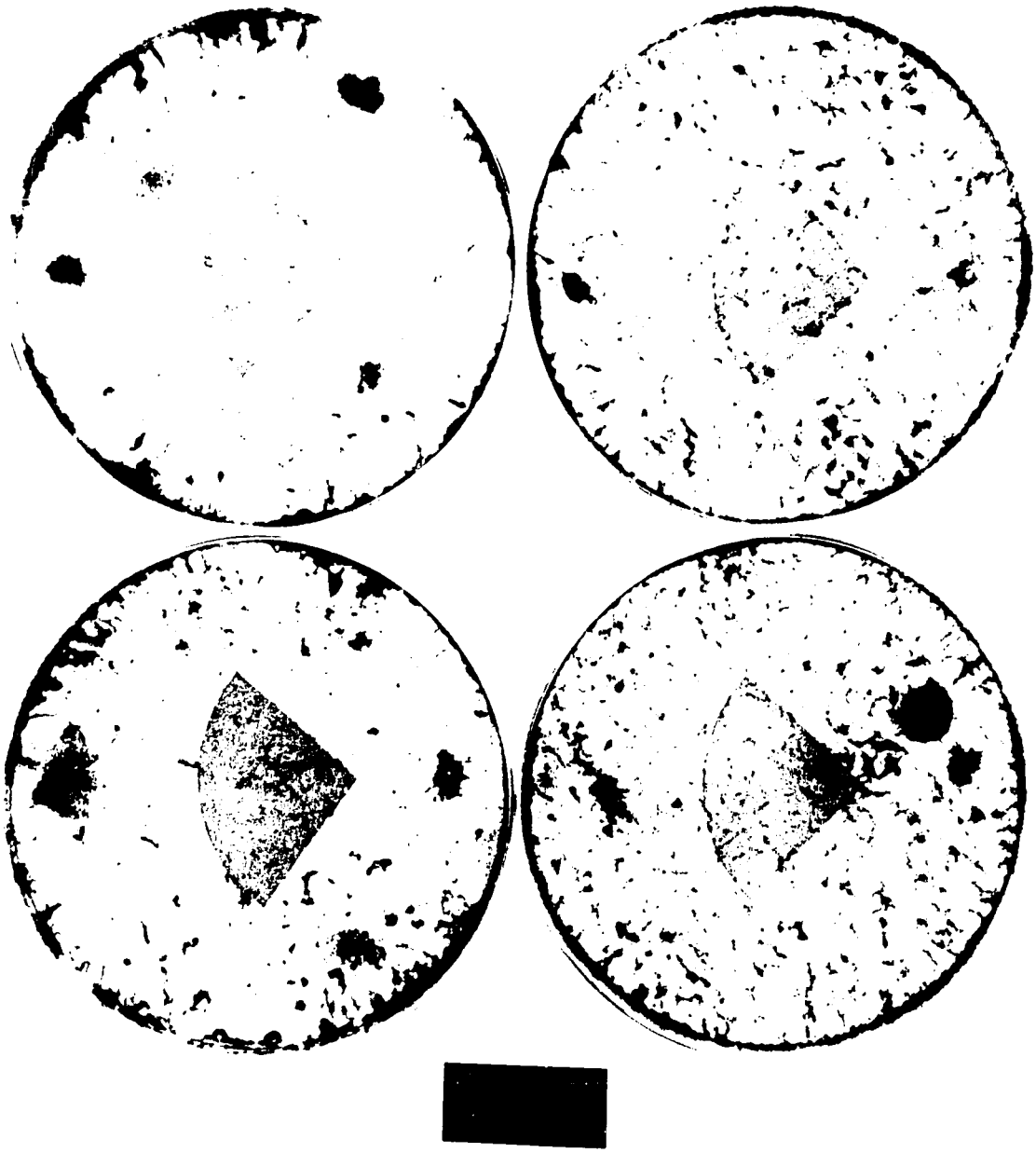


PLATE III.



Plates IV - V. Photographs showing the dose effect of the extract of the cross Em A x Em a on single wild-type strain cultures (Em A and Em a), when compared with untreated control (upper left corner).

PLATE IV.

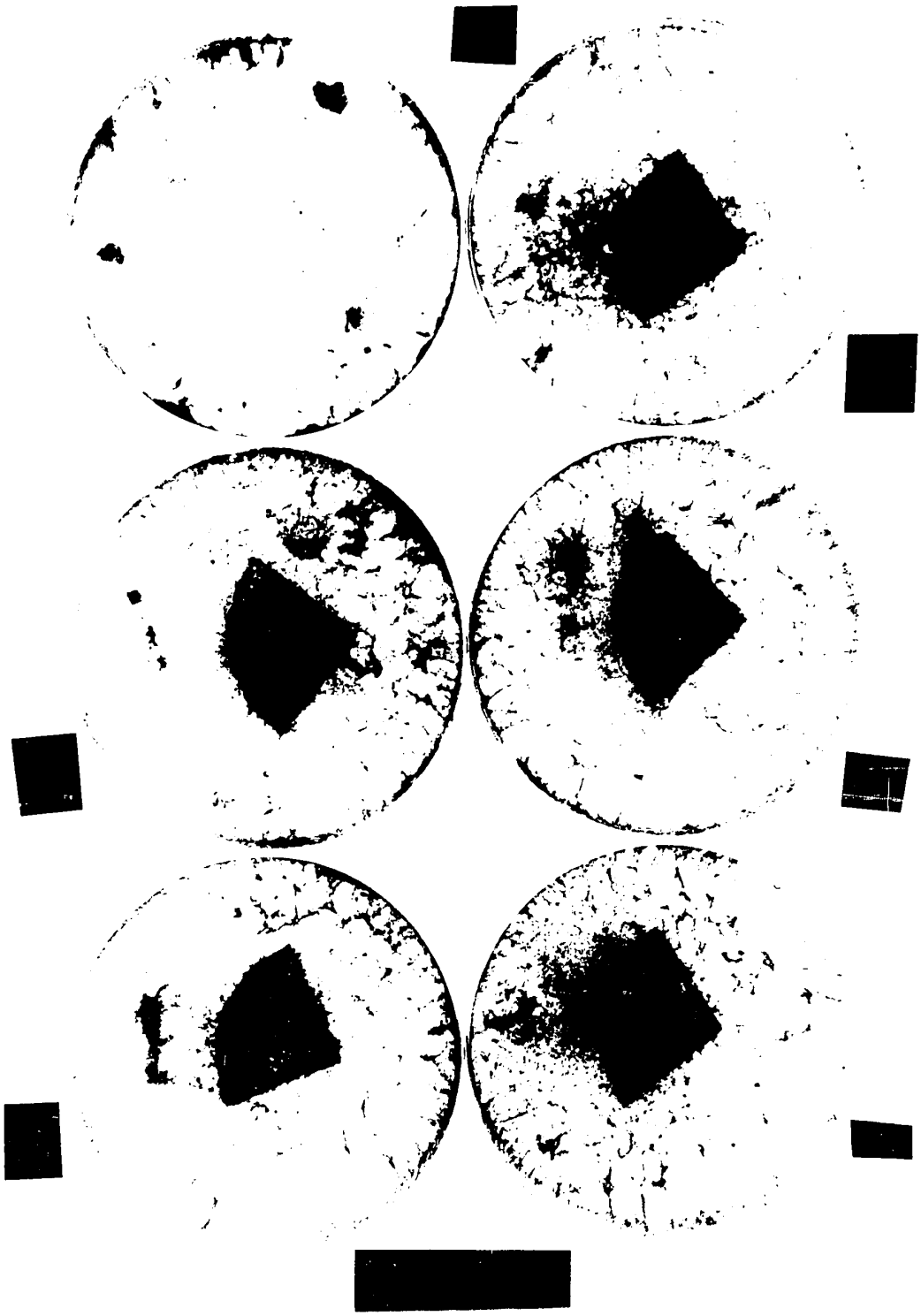
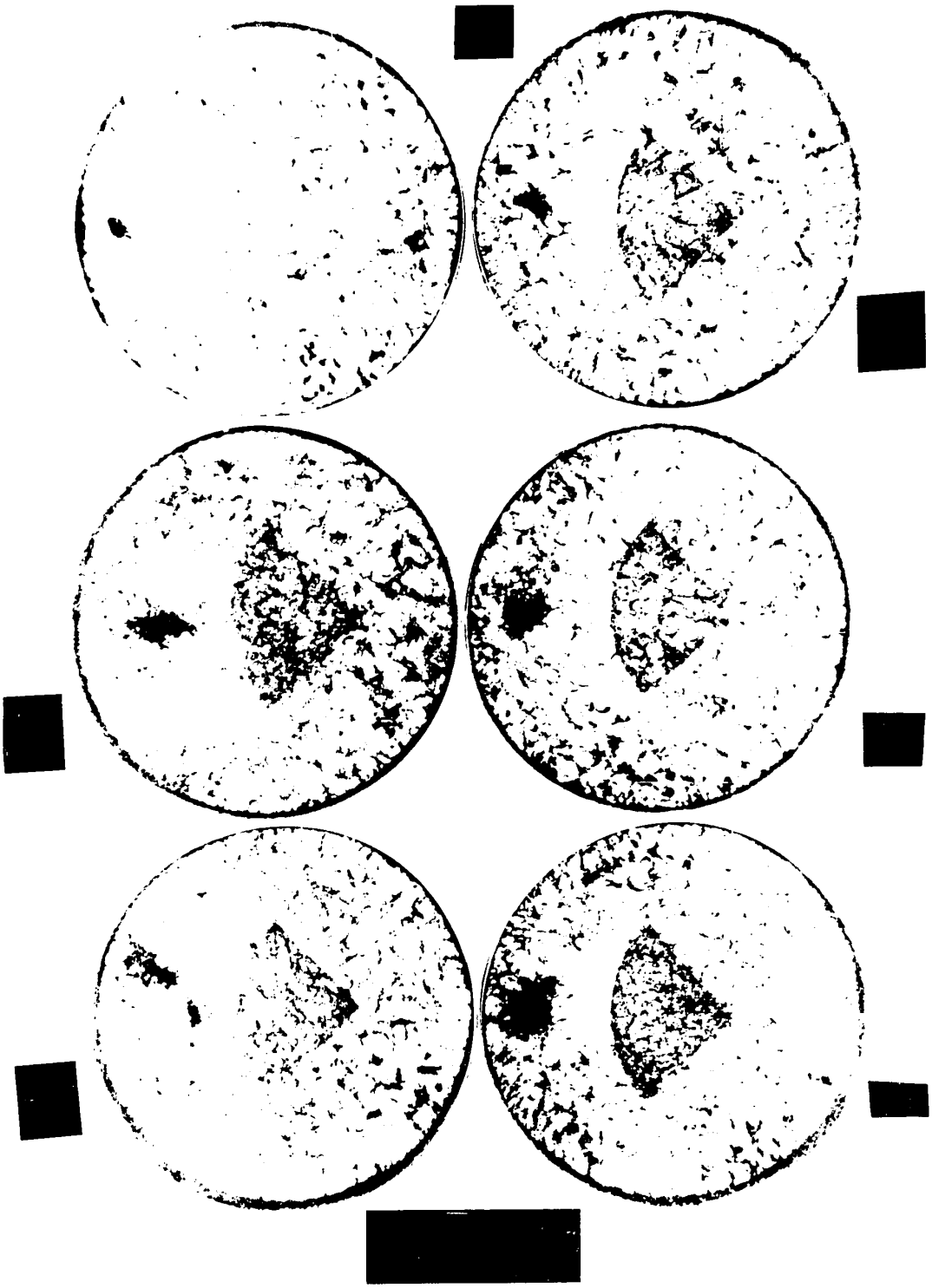


PLATE V.



Plates VI - VII. Photographs showing the effect of the extract (Plate VI) of the cross Em A x Em a on a cross I-5 (sterile) x Em a (wild), when compared with the untreated control (Plate VII). I-5 (sterile) is an isolate from the 'selfed' progeny of Em A.

PLATE VI.

PLATE VII.

Plates VIII - IX. Photographs showing the effect of the extract of cross Em A x Em a on isolate #I-28 (Bisexual, self-sterile (Plate VIII)), when compared with the untreated control (Plate IX).

PLATE VIII.

EXTRACT EmA X Emm

PLATE IX.

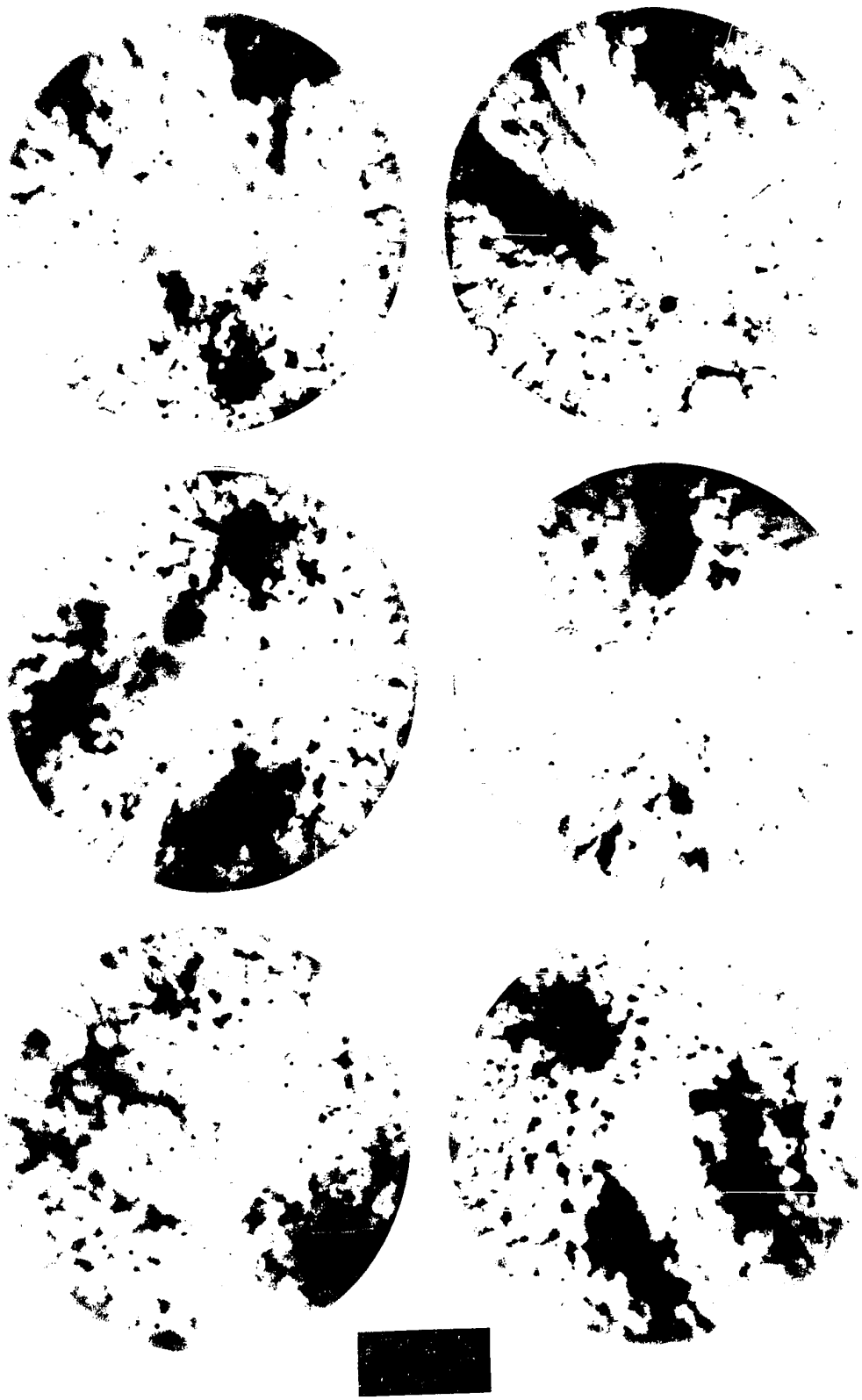
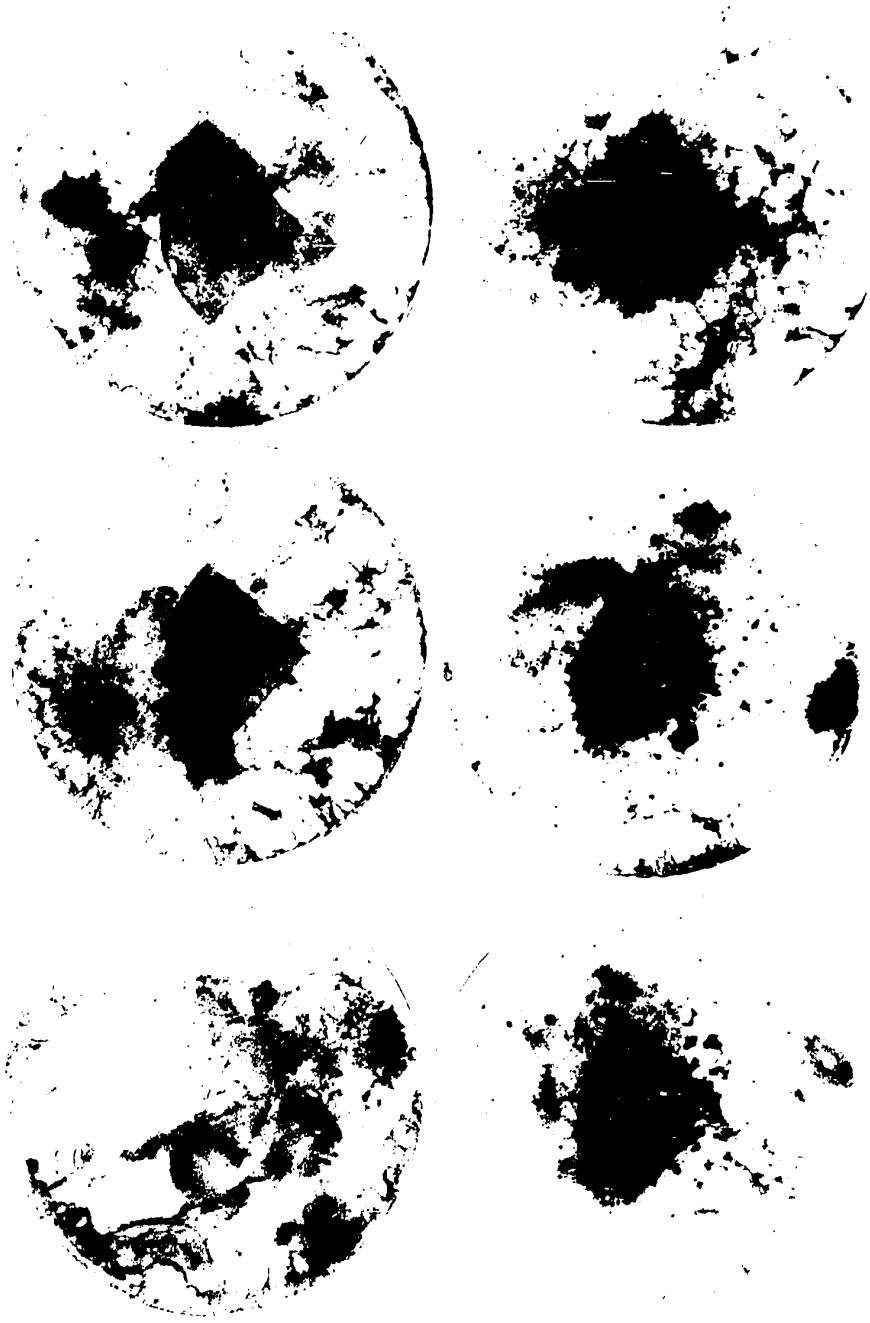


Plate X. Photograph showing the effect of the extract of cross Em A X Em a on a biochemical mutant of hist-2A, when compared with untreated control.

PLATE X.

His-2 'A'

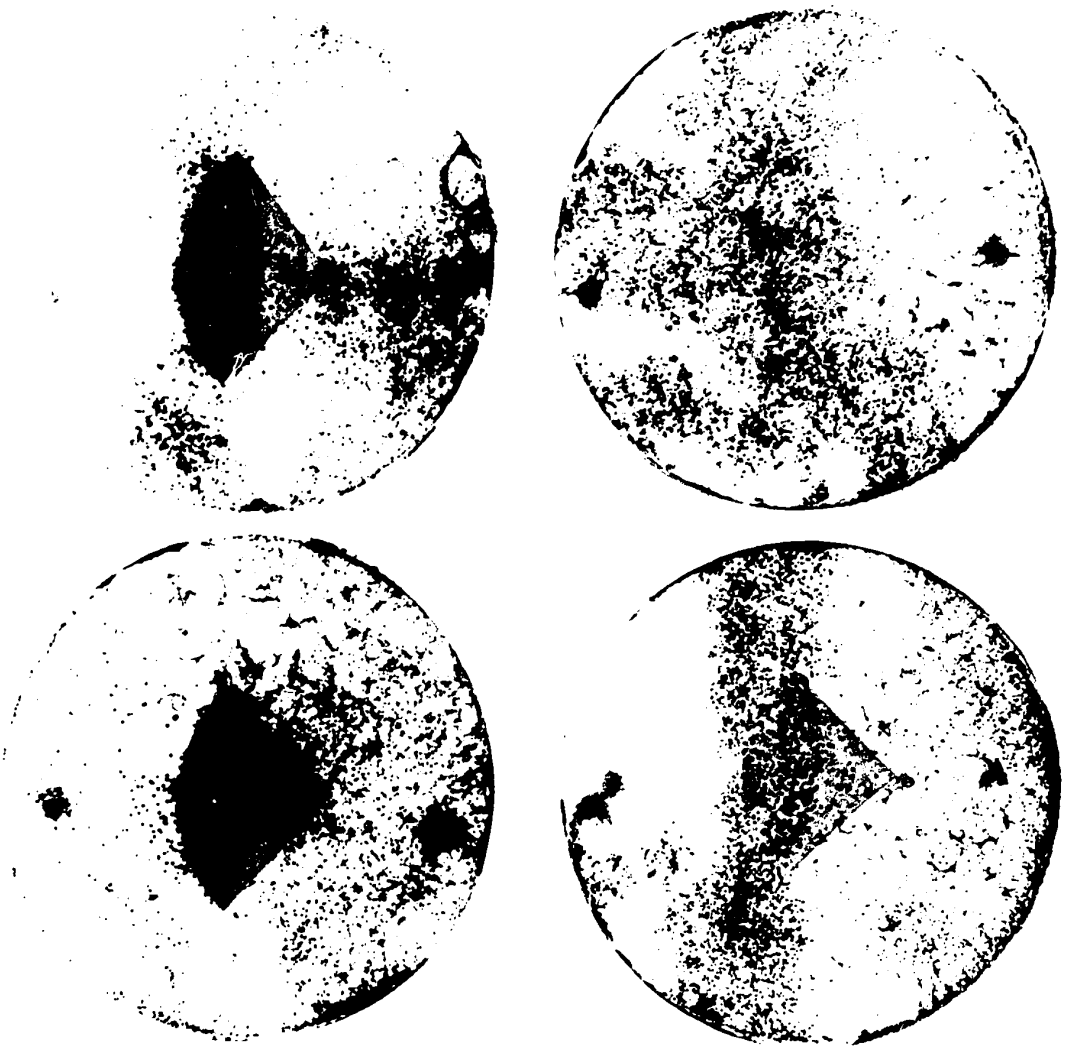


CONTROL

CYPRAC™ EmA X Emn

Plate XI. Photograph showing the effect of the extract of cross Em A x Em a on the cross Em A x Em a (wild types), when compared with the untreated control.

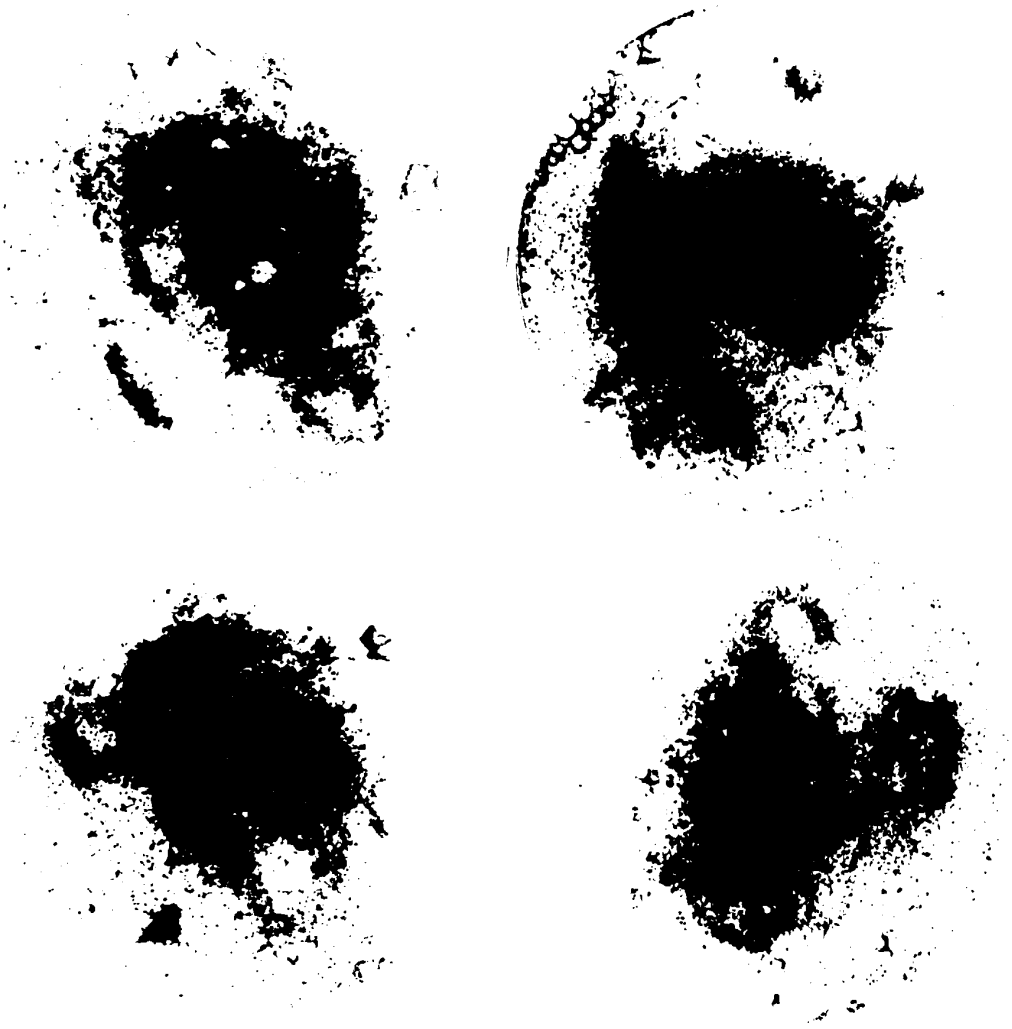
PLATE XI



Plates XII - XIII. Photographs showing the effect of the extract of cross Em A x Em a on a cross 5366-A (sterile) x Em a (Plate XII), when compared with the untreated control (Plate XIII).

PLATE XII.

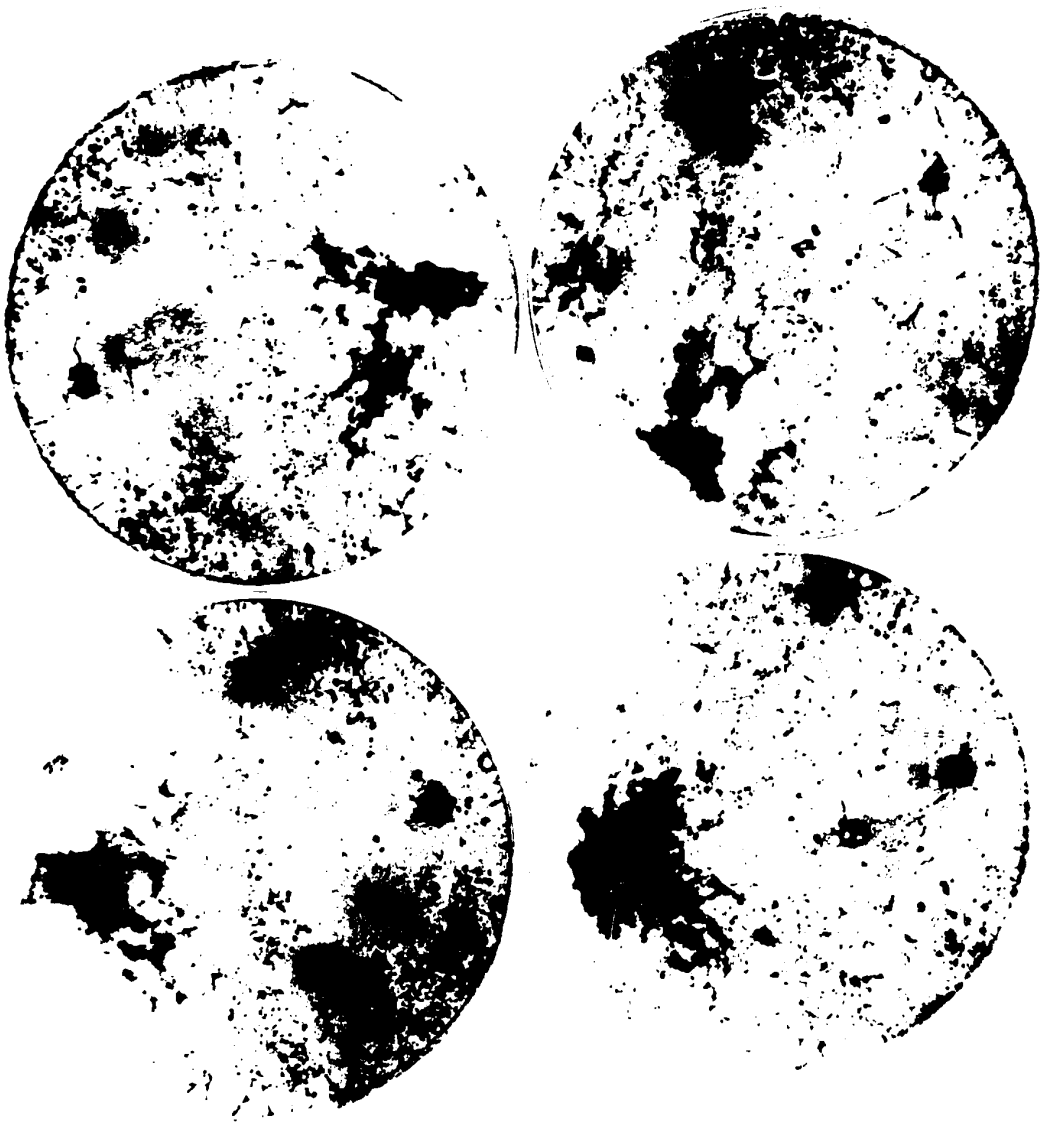
5311-A x Emc



[redacted]

PLATE XIII.

5311-A x Emo



[redacted]

Plates XIV - XV. Photographs showing the effect of the extract of cross Em A x Em a on the cross 7232-A (sterile) x Em a (Plate XIV) and 10710-A x Em a (Plate XV), when compared with the untreated control.

PLATE XIV.

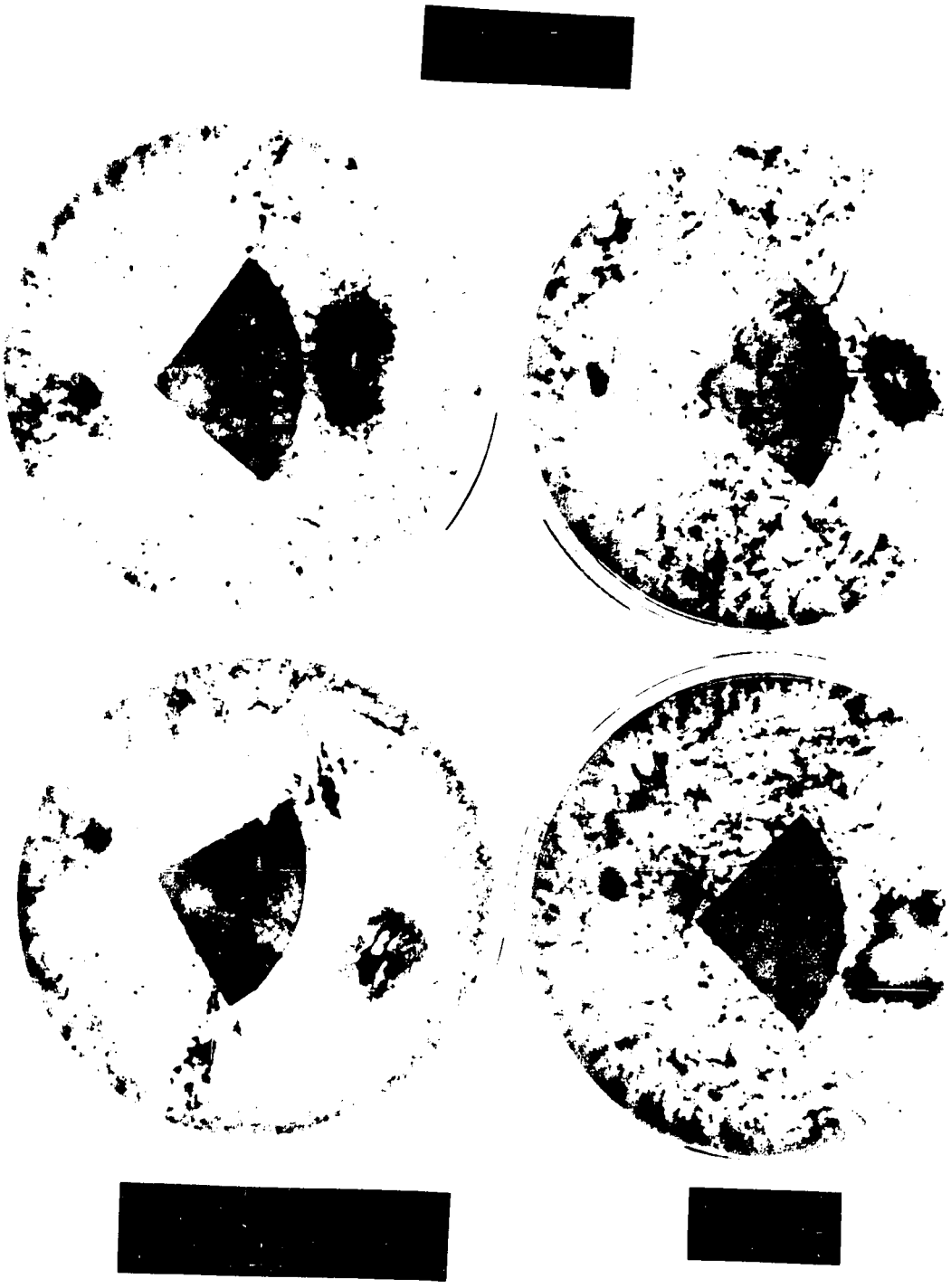


PLATE XV.

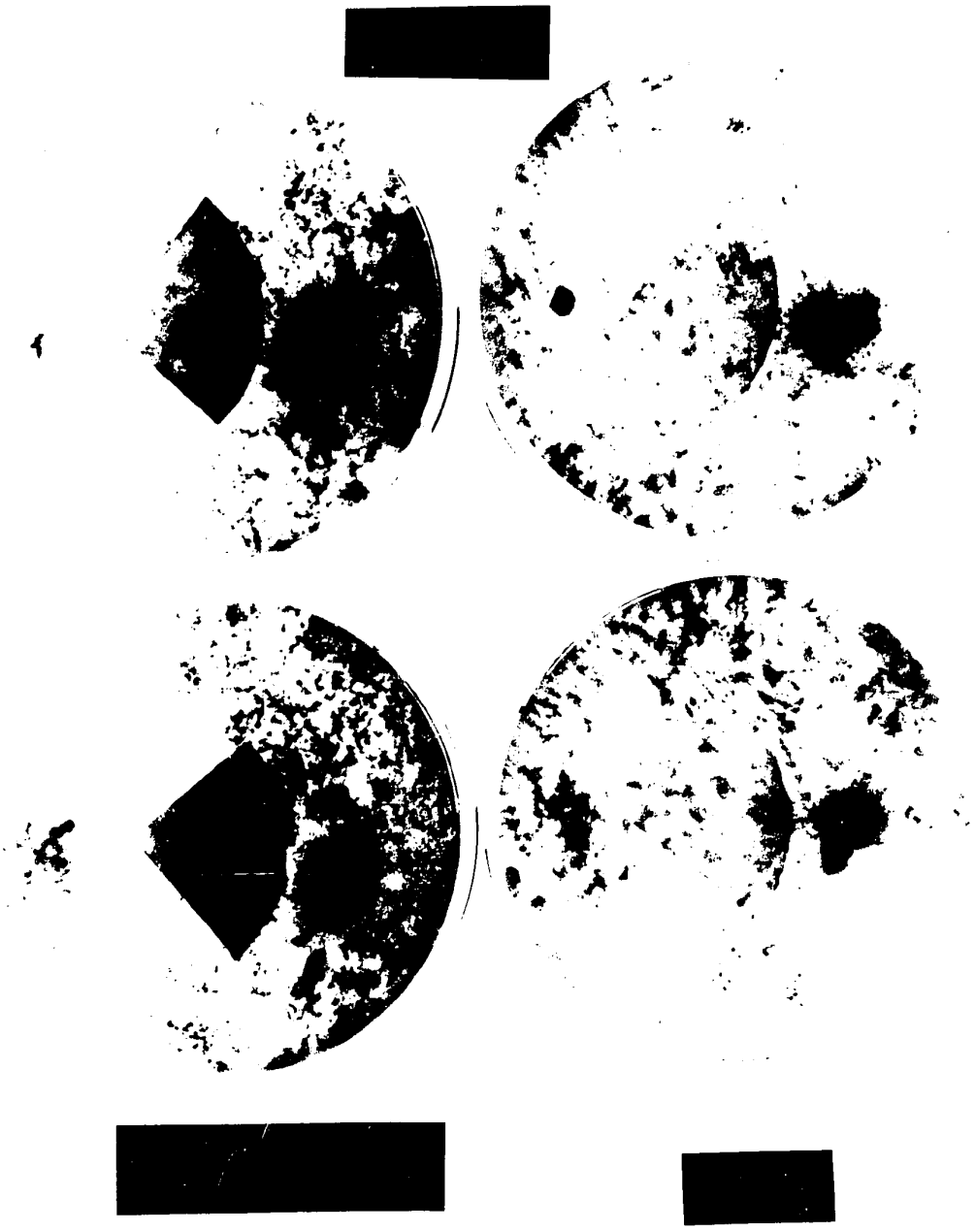


Plate XVI. Photograph showing the effect of the extract of cross Em A x Em a on the cross 9312-A (sterile) x Em a, when compared with the untreated control.

PLATE XVI.

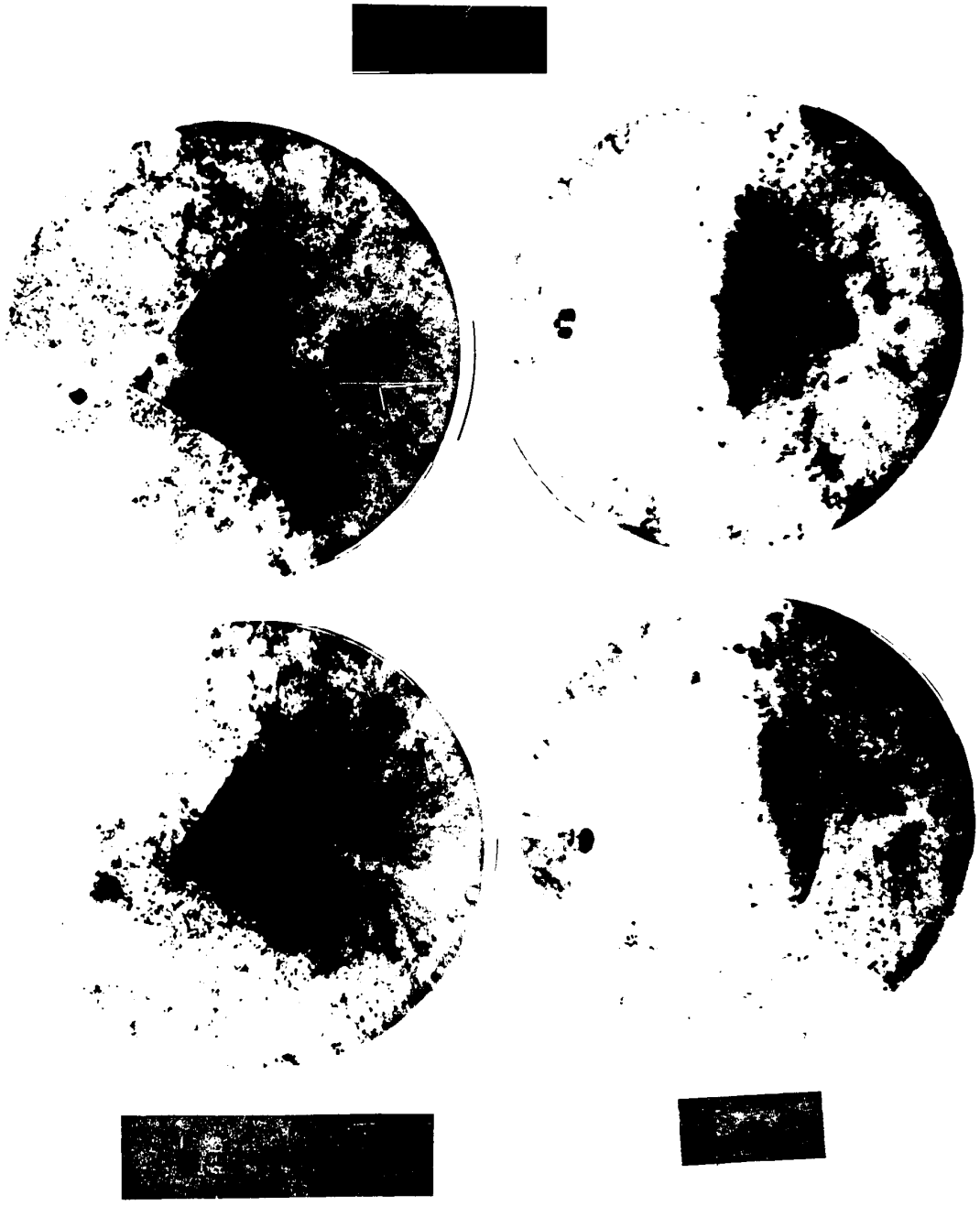


Plate XVII. Photograph showing the effect of the extract of cross Em A x Em a on the cross 10402-A (semi-sterile) x Em a, when compared with the untreated control.

PLATE XVII.

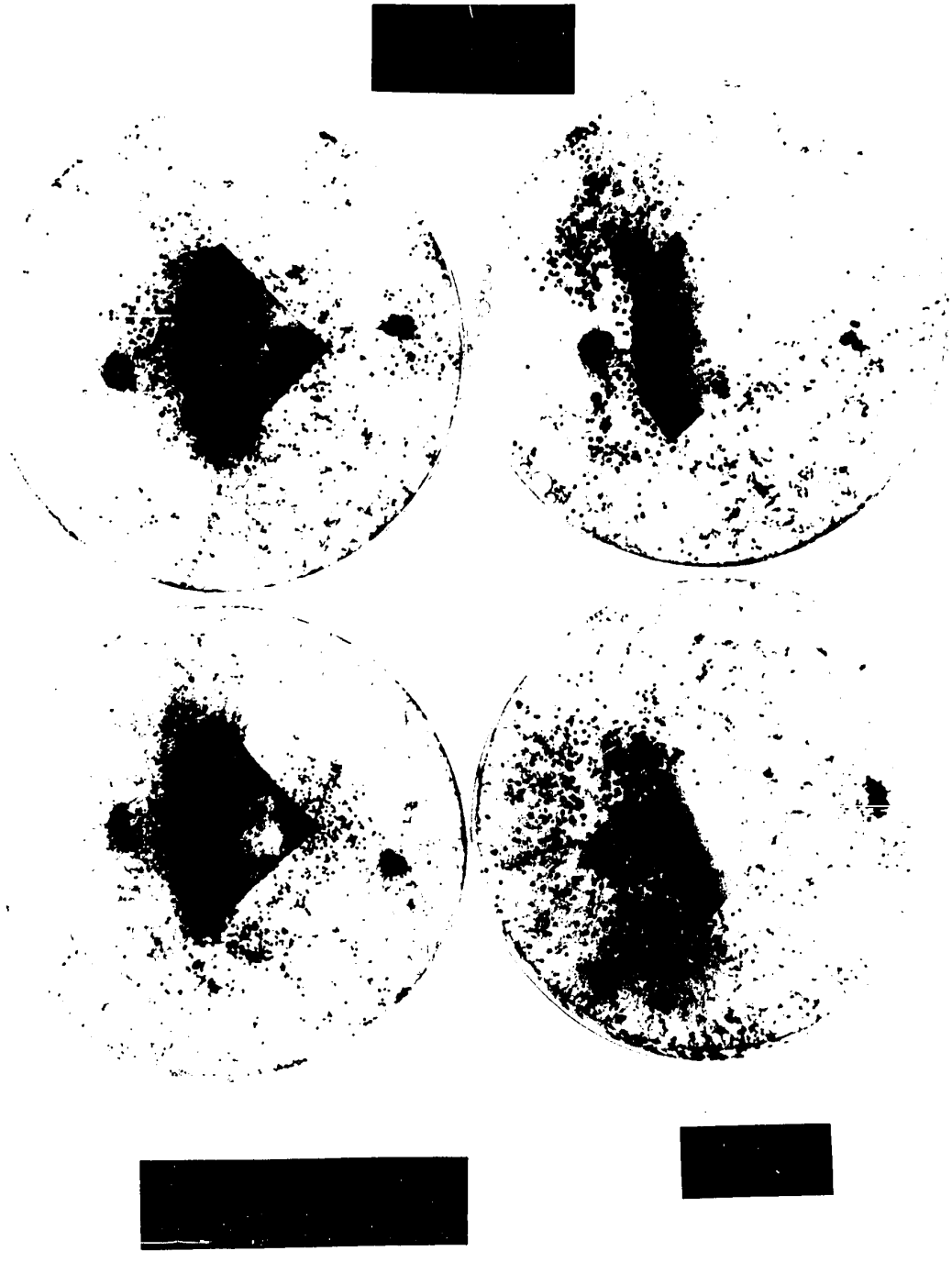


Plate XVIII. Photograph showing the separation of compounds of the cross extract (Em A x Em a) by thin-layer chromotography with a 3:1 (v/v) chloroform-benzene solvent system. The arrow indicates the direction of the flow of the solvent. The fluorescence of the compounds under UV light of 2540Å has been marked accordingly - B = Blue, LB = Light Brown. Origins marked 1 and 2.

PLATE XVIII.


B
LB
LE
2
MAX Encl

Plates XIX - XX. Photographs showing the effect of the extracts of single strains Em A or Em a on wild-type strain Em A (Plate XIX), when compared with untreated control (Plate XX).

PLATE XIX.

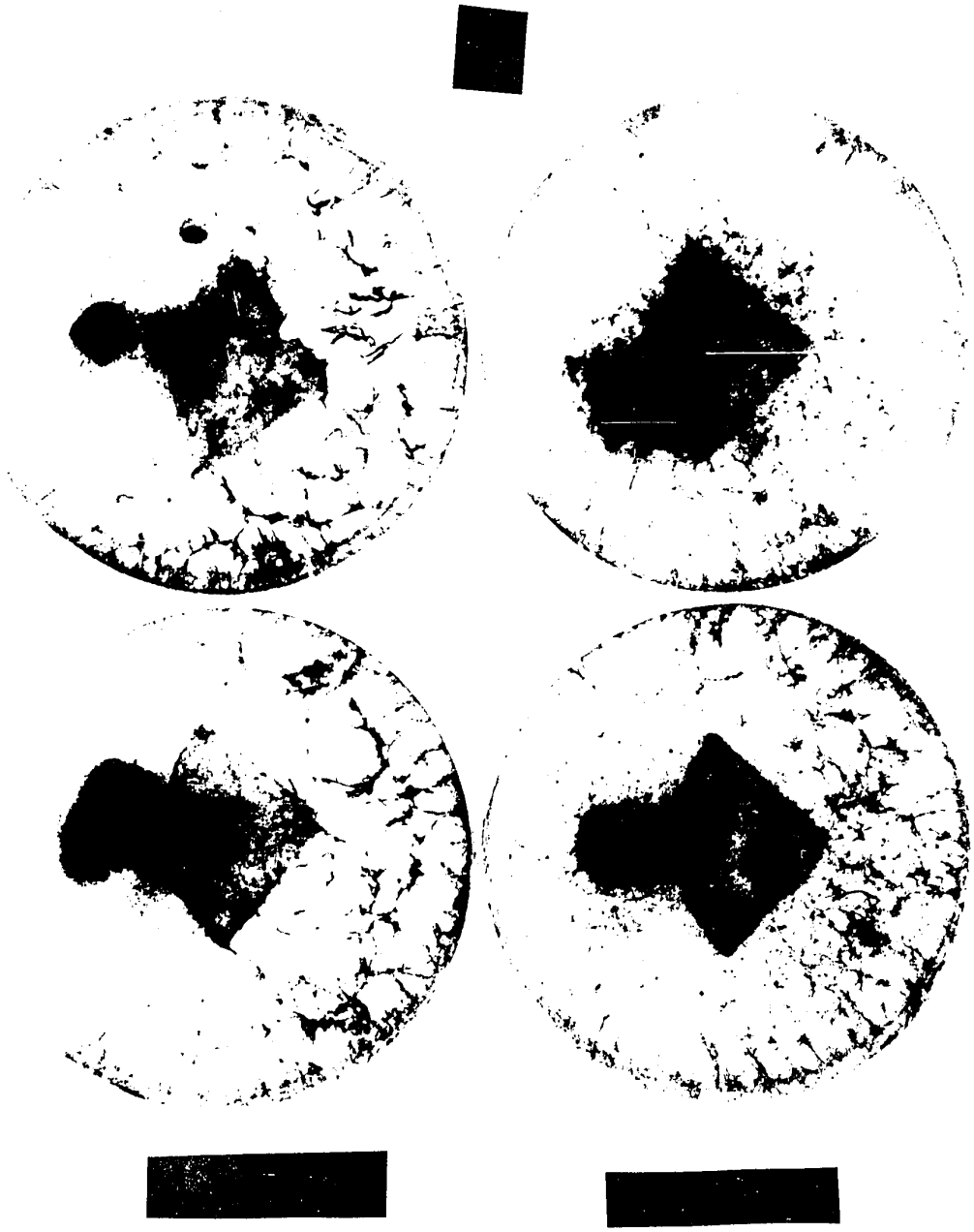
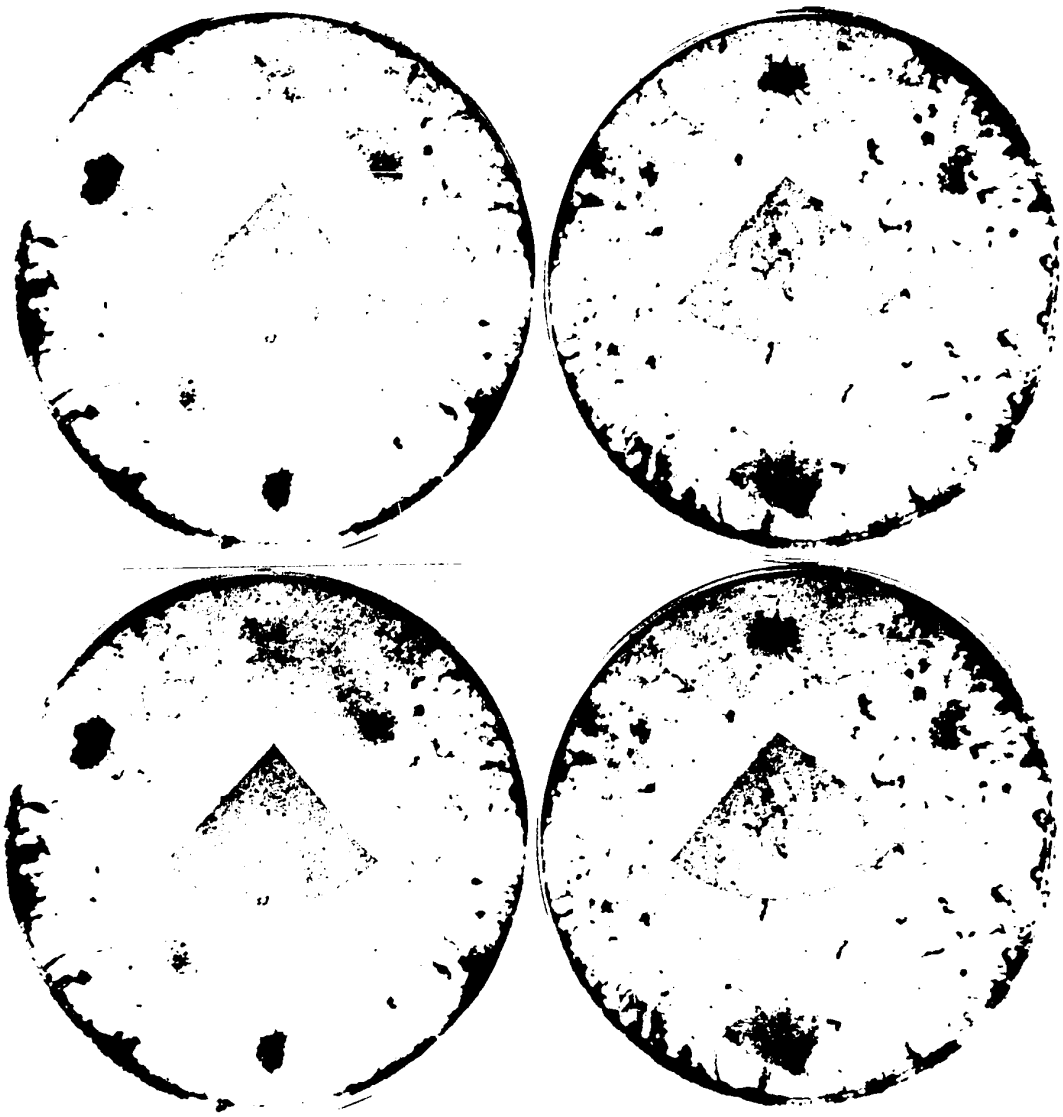


PLATE XX.



Plates XXI - XXII. Photographs showing the effect of the extracts of single strains Em A or Em a on the cross Em A x Em a (Plate XXI), when compared with untreated control (Plate XXII).

PLATE XXI.

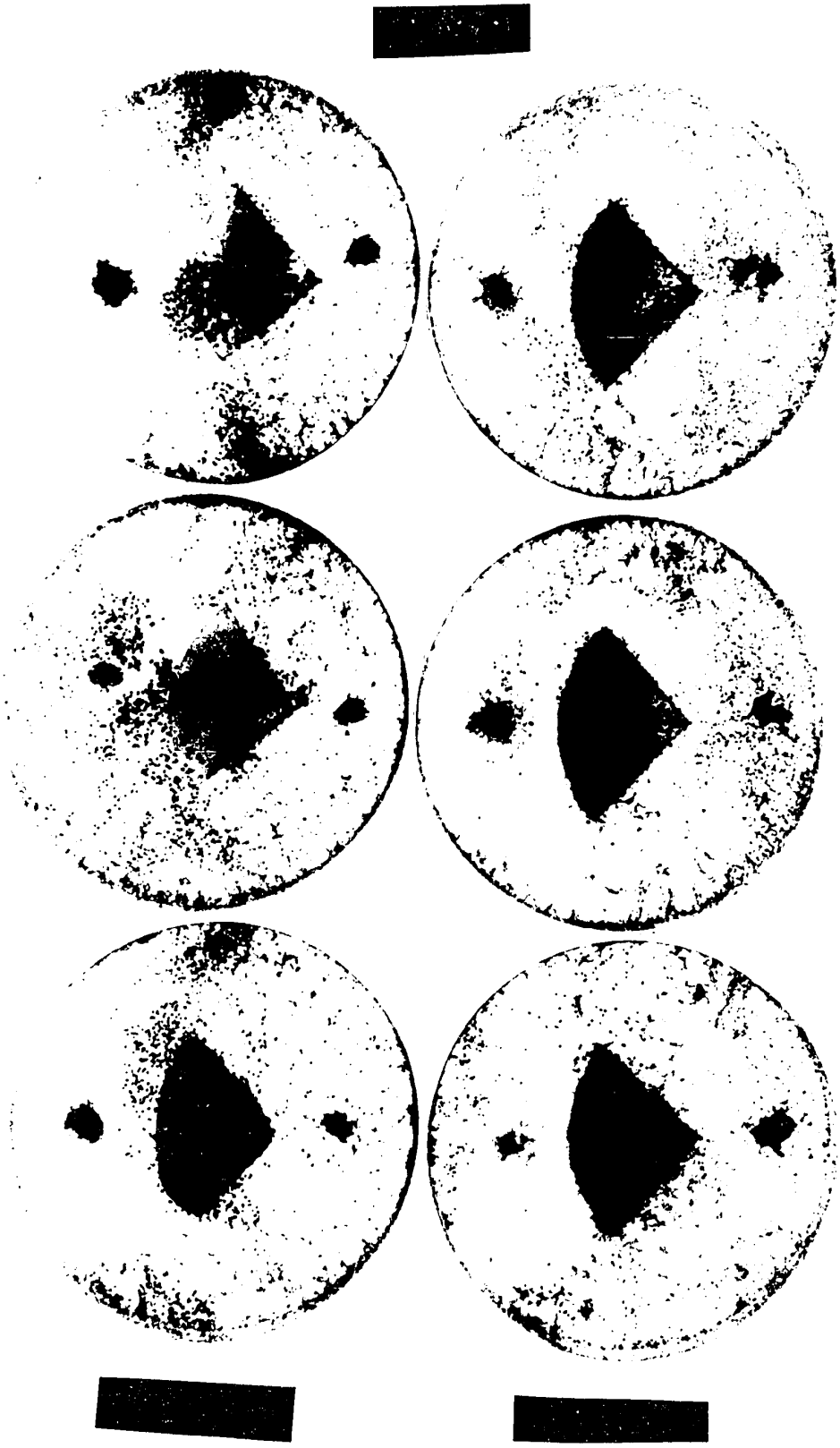
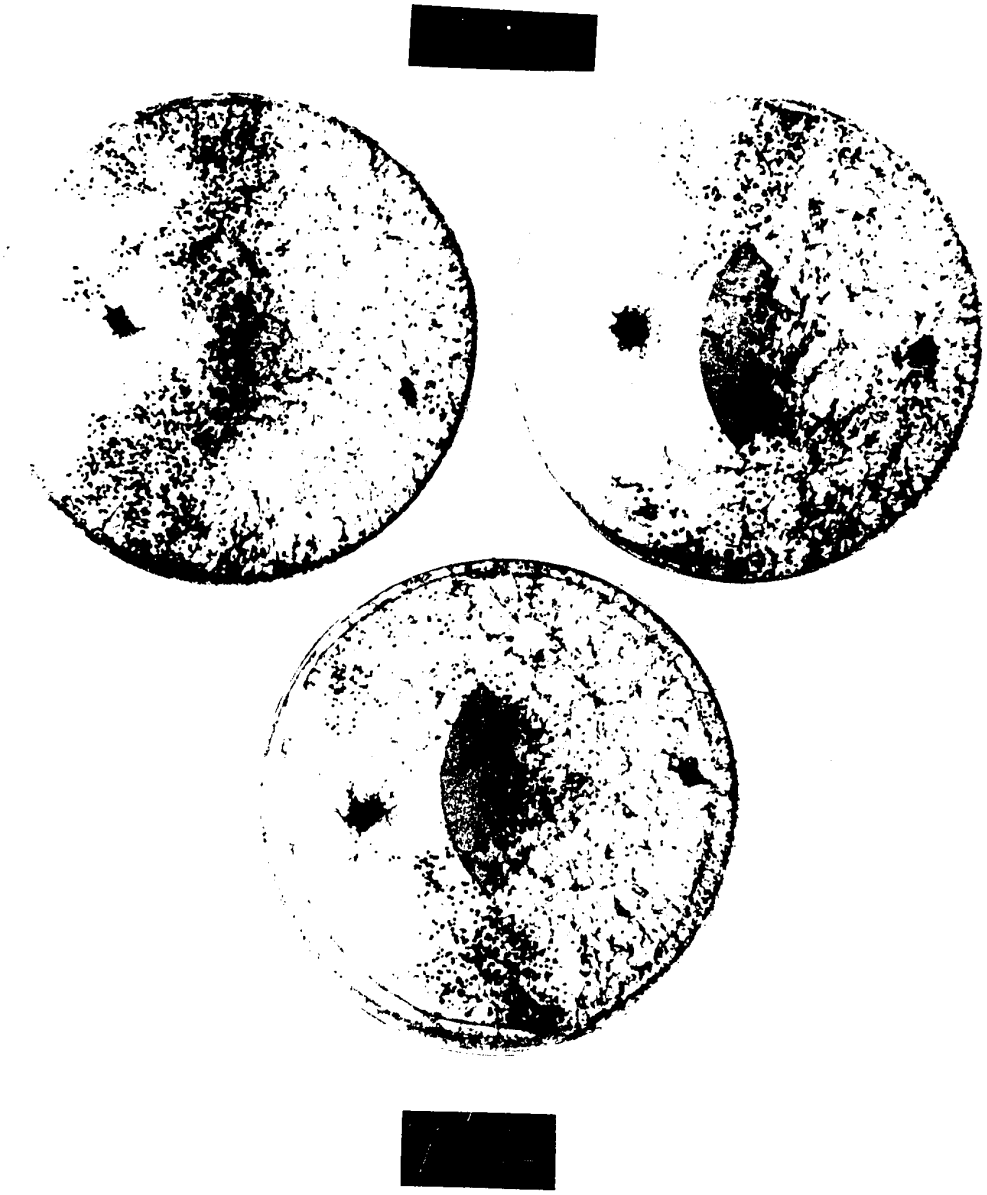


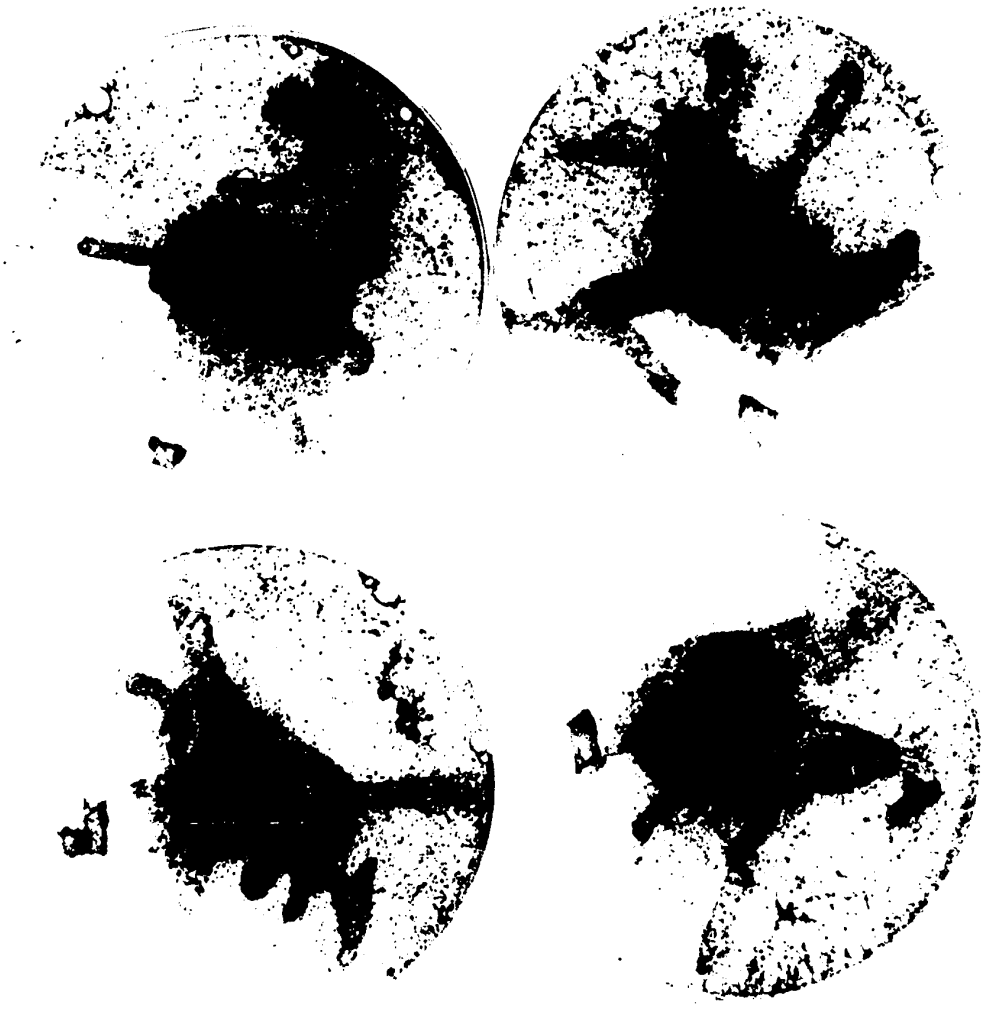
PLATE XXII.



Plates XXIII - XXV. Photographs showing the effect of extracts of single strains (Em A or Em a) on the cross 5366-A (sterile) x Em a (Plates XXIII and XXIV), when compared with untreated control (Plate XXV).

PLATE XXIII.

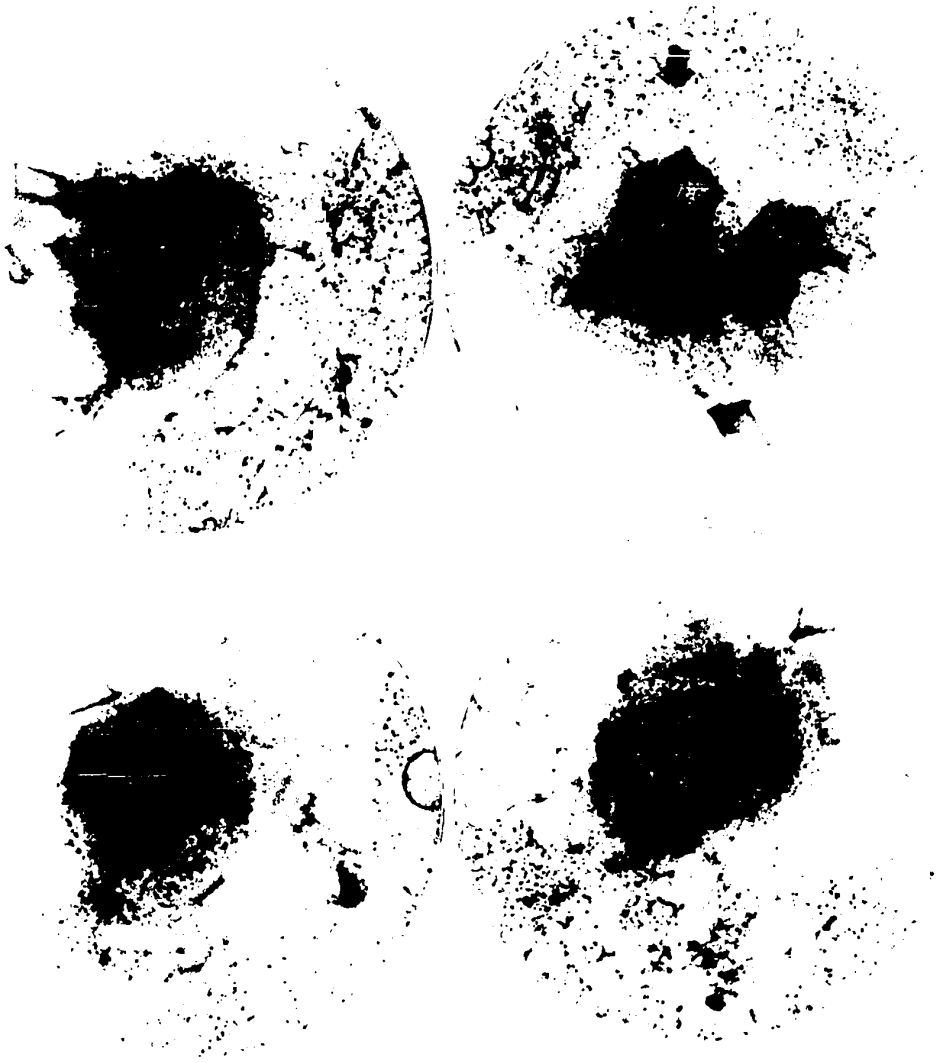
5311-A x Emo



[redacted]

PLATE XXIV.

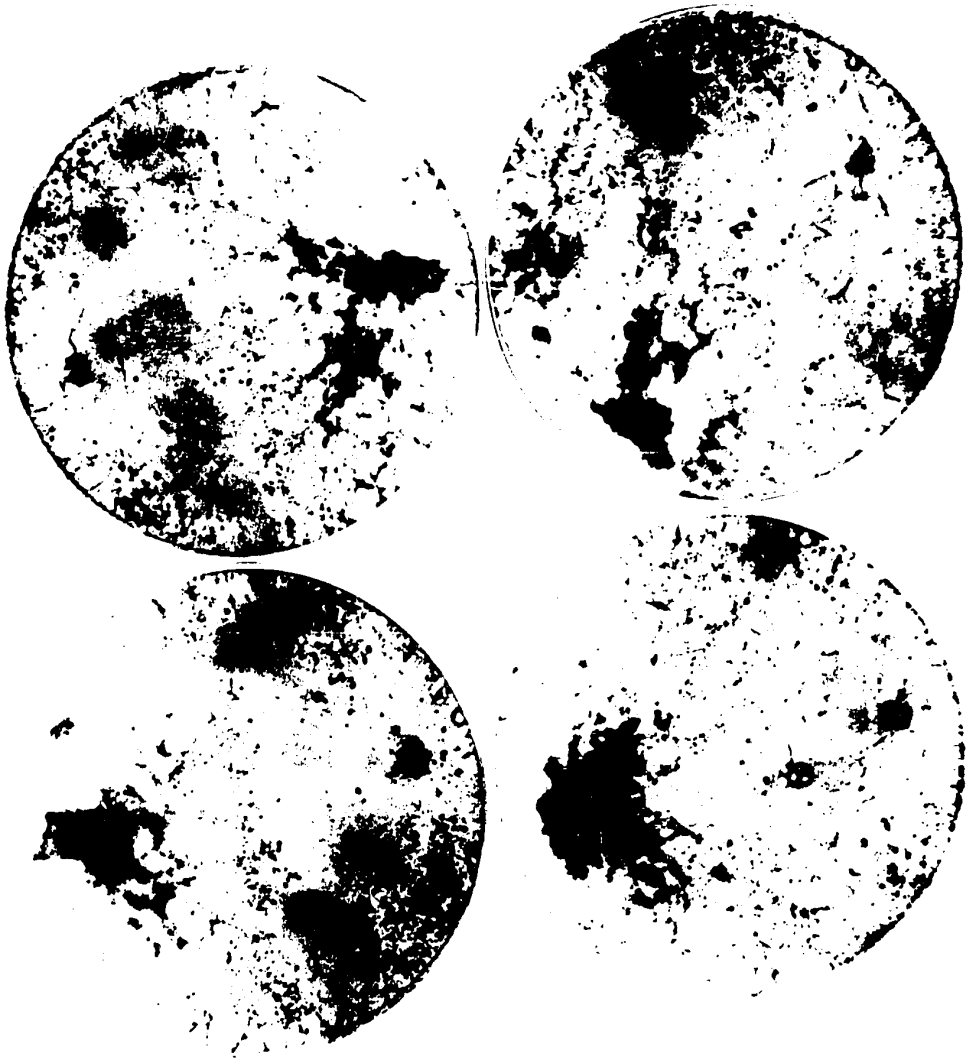
5311-A x Emc



[redacted]

PLATE XXV.

5311-A x Emc



[redacted]

Plate XXVI. Photograph showing the effect of extracts of single strains (Em A or Em a) on the cross 7232-A (sterile) x Em a, when compared with untreated control.

PLATE XXVI.

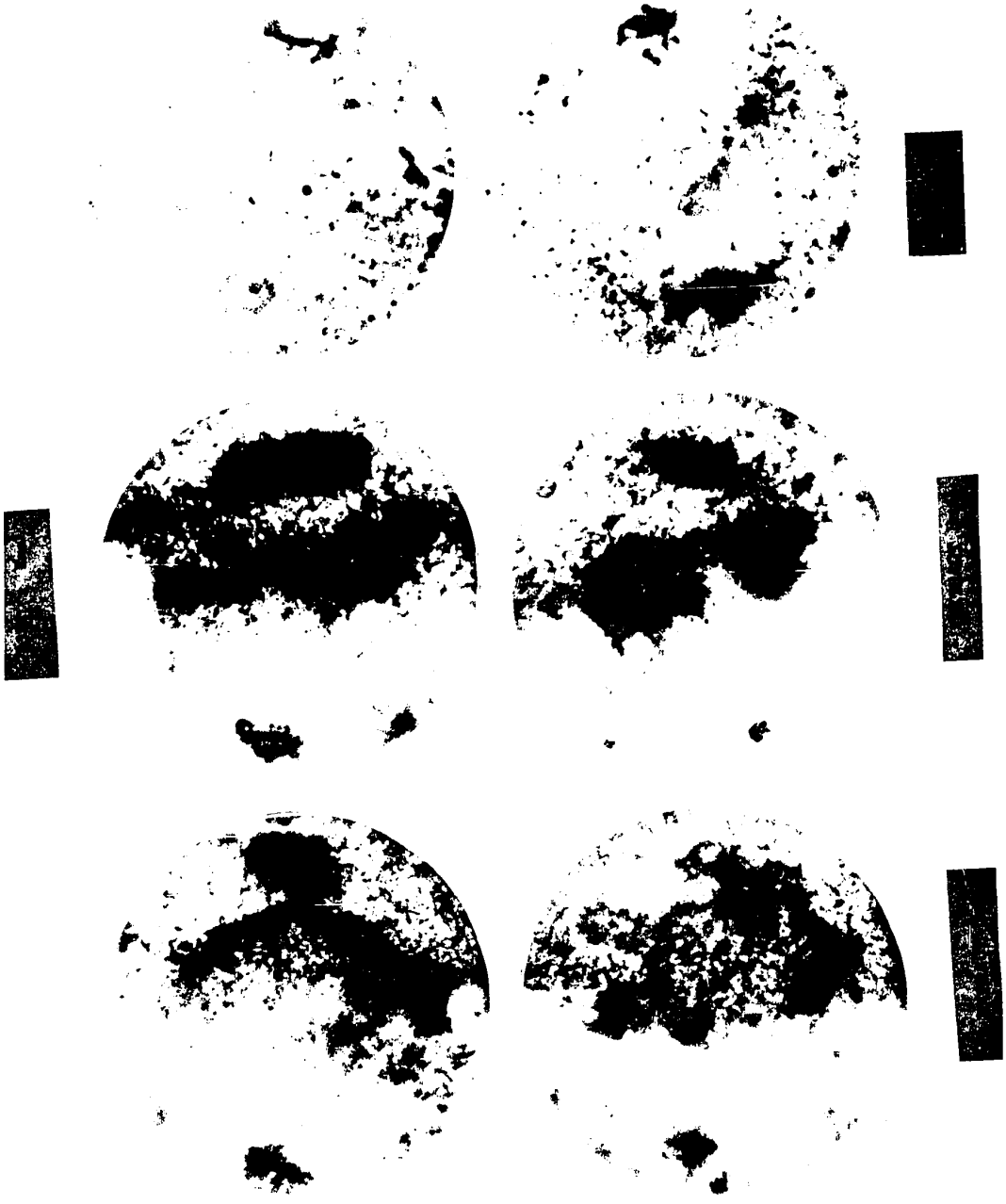


Plate XXVII. Photograph showing the effect of extracts of single strains (Em A or Em a) on the cross 10710-A (sterile) x Em a, when compared with untreated control.

PLATE XXVII.

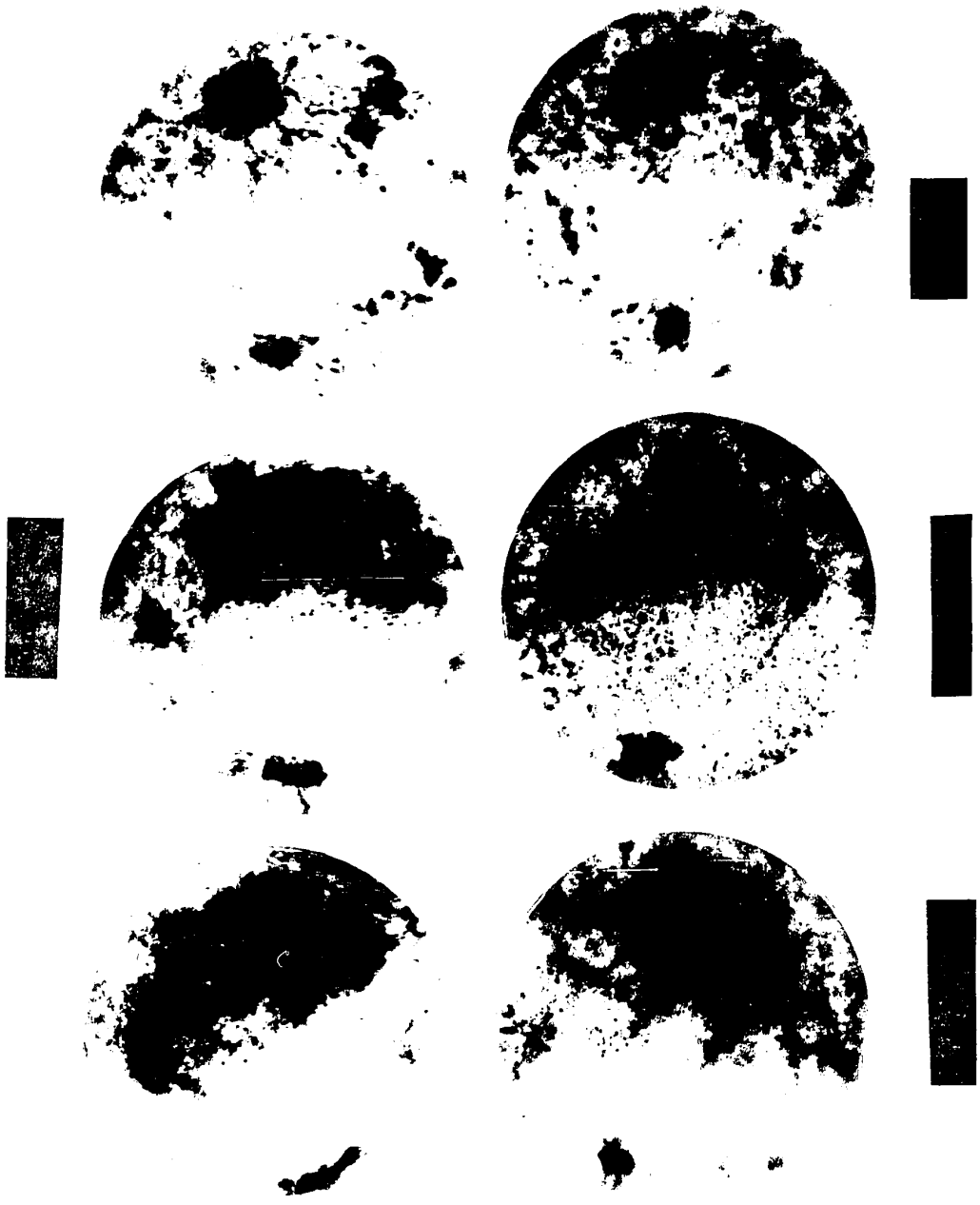
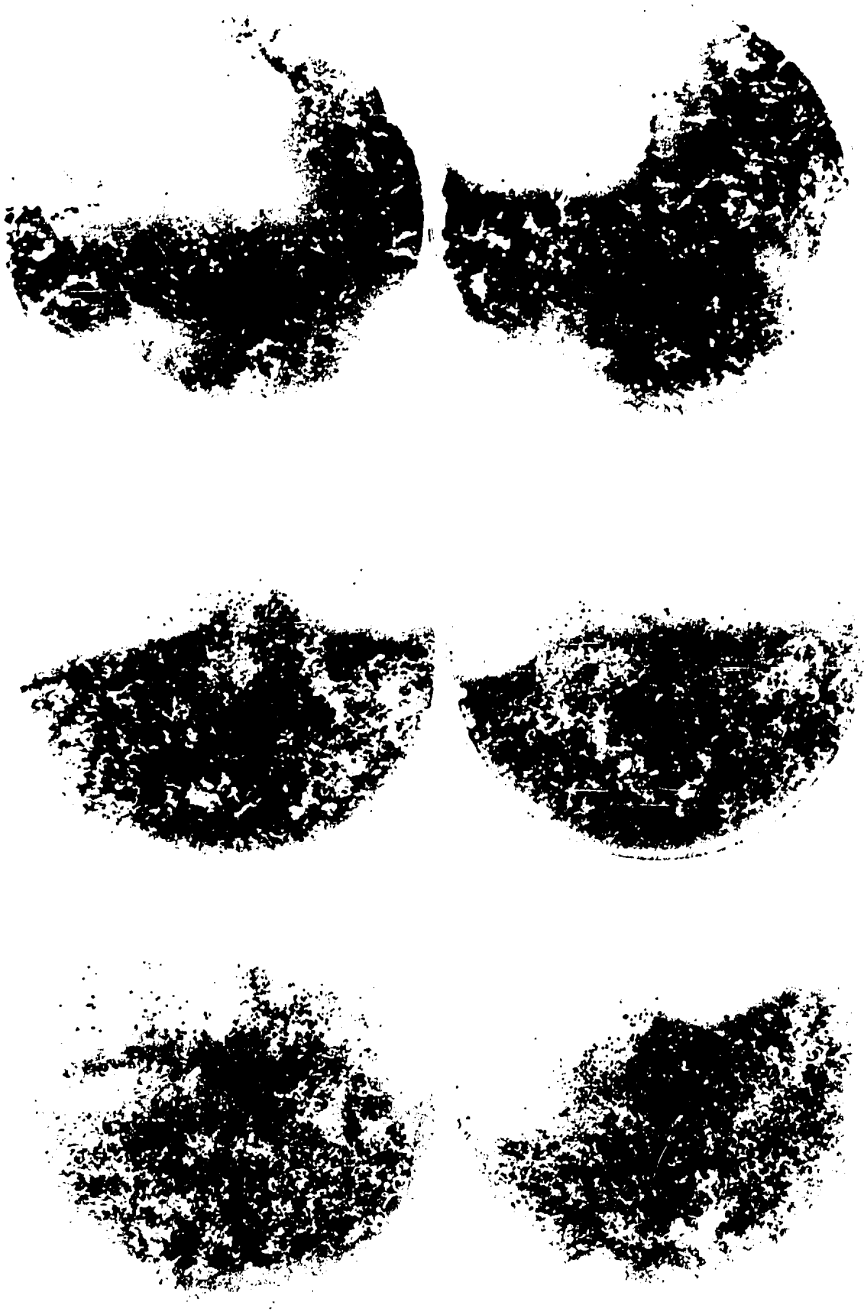


Plate XXVIII. Photograph showing the effect of extracts of single strains (Em A or Em a) on the cross 9312-A (sterile) x Em a, when compared with untreated control.

PLATE XXVIII.

Plate XXIX: Photograph showing the effect of extracts of single strains (Em A or Em a) on the cross 10402-A (semi-sterile) x Em a, when compared with untreated control.

PLATE XXIX.



Plates XXX - XXXII. Photographs showing the effect of the extracts of the single strains Em A (Plate XXX) and Em a (Plate XXXI) on the cross I-5 (sterile) x Em a, when compared with untreated control (Plate XXXII).

PLATE XXX.

[redacted]

[redacted]

PLATE XXXI.

PLATE XXXII.

Plate XXXIII. Photograph of a thin-layer chromatogram of single strain extracts (Em A and Em a). The solvent system utilized was 3:1 chloroform-benzene solution (v/v). The arrow indicates the direction of the flow of the solvent. The fluorescene of the developed spots were marked by pencil under UV light of 2540Å.

B = Blue

PLATE XXXIII.


B
B
B
B
1
2
EmA

Plate XXXIV. Photograph of the UV spectrogram of single strain extracts of Em A and Em a.

PLATE XXXIV.

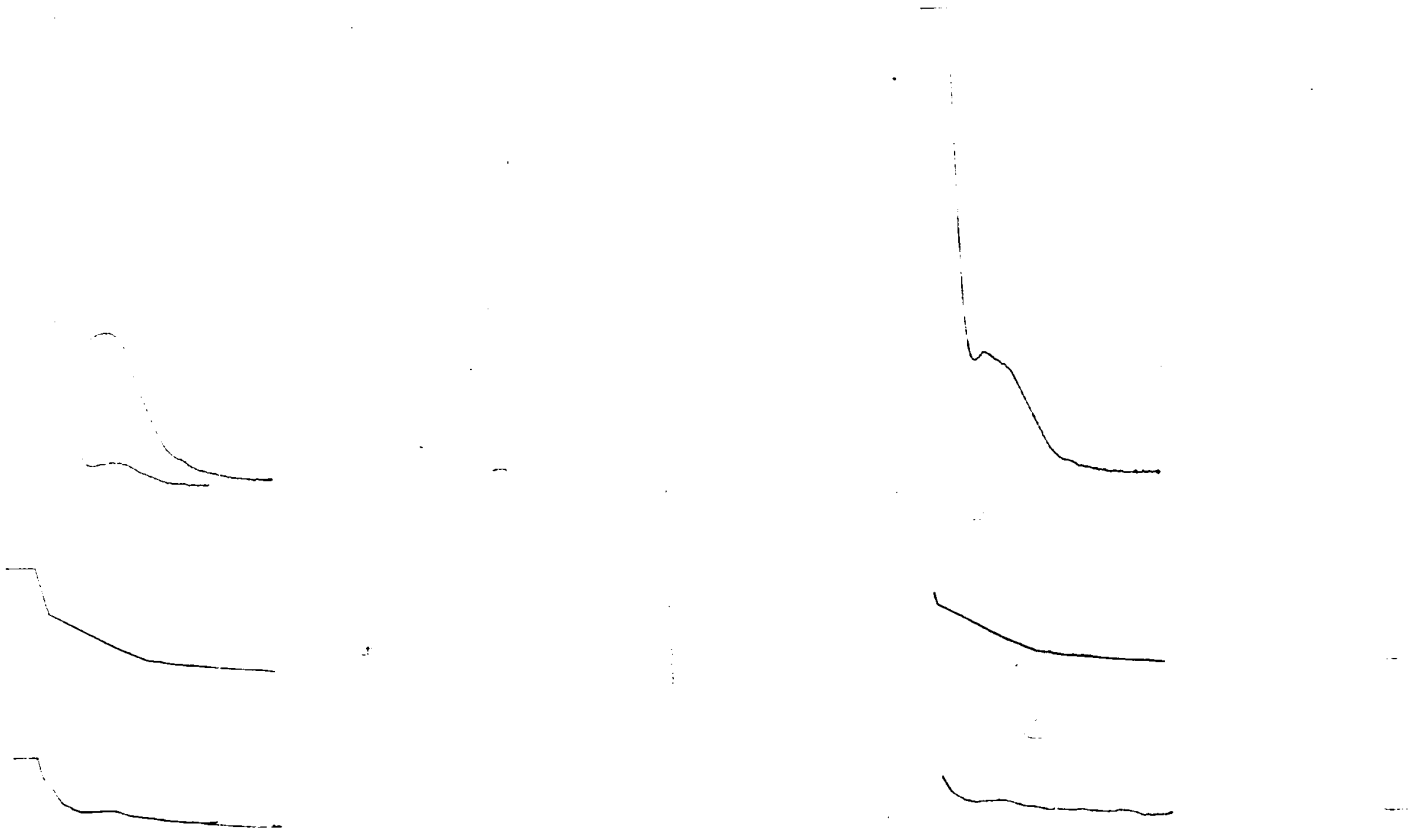
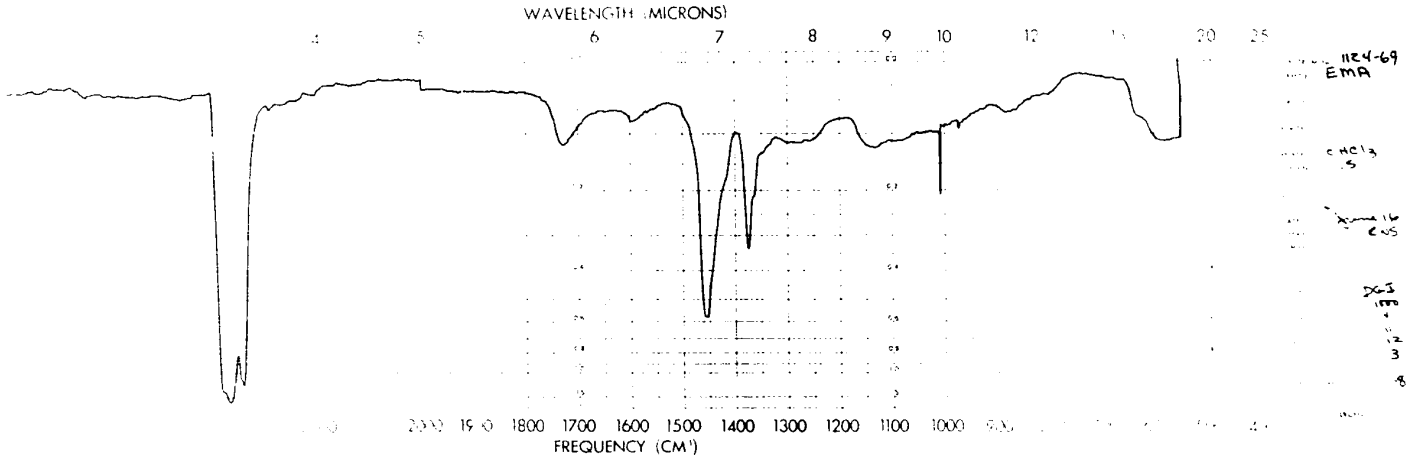


Plate XXXV. Photograph of the IR spectra of the single strain extracts of Em A and Em a.

PLATE XXXV.


WAVELENGTH (MICRONS)
6
7
8
9
10
FREQUENCY (CM⁻¹)
1800 1700 1600 1500 1400 1300 1200 1100 1000
1124-69
EMA

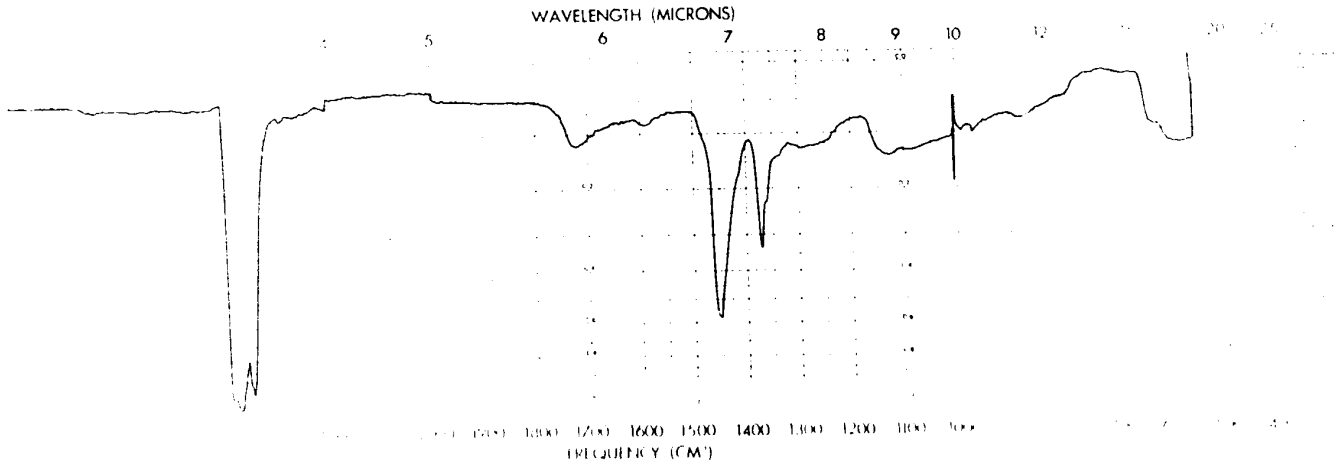
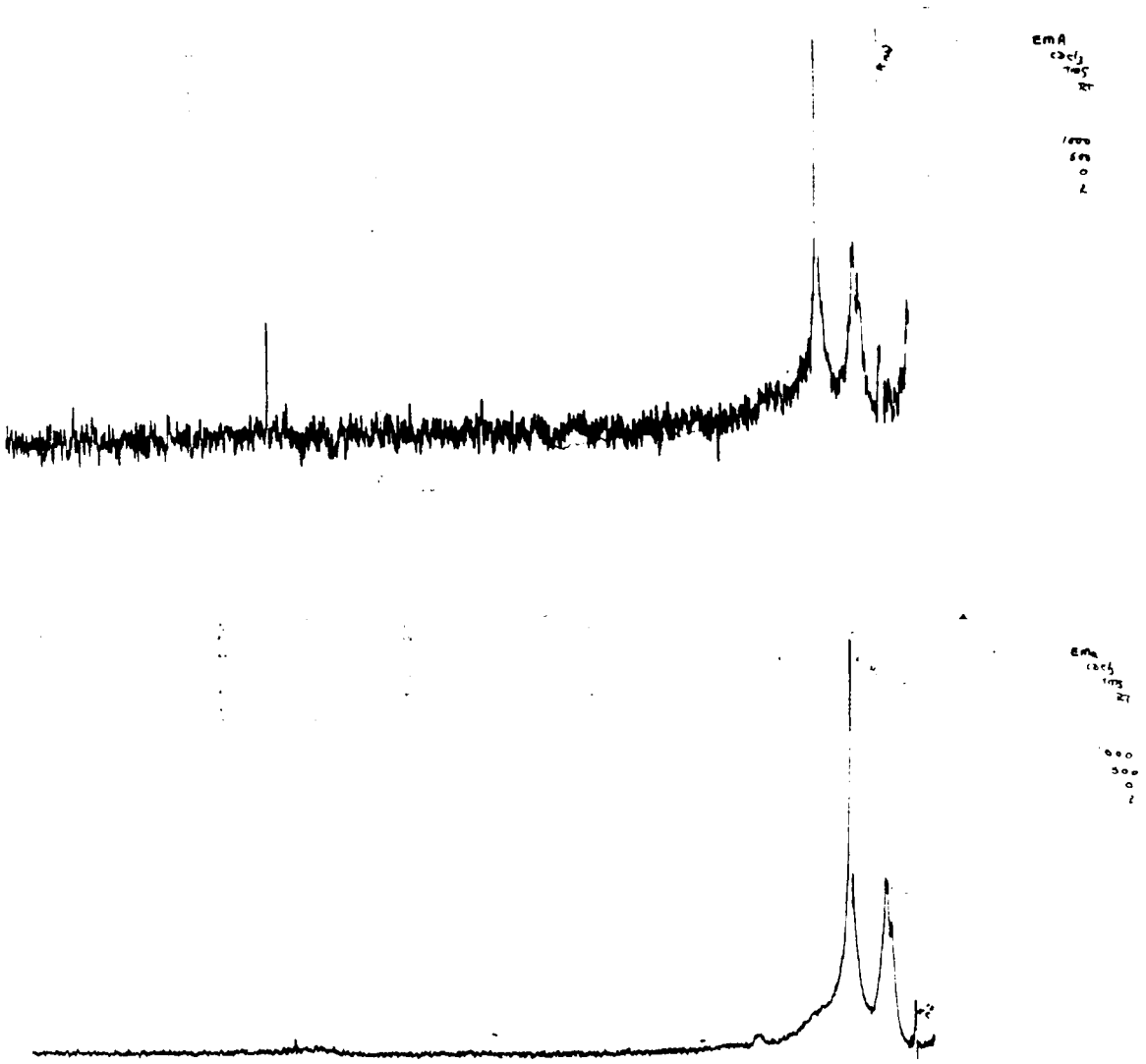

WAVELENGTH (MICRONS)
6
7
8
9
10
1600 1500 1400

Plate XXXVI. Photograph of the NMR spectra of the single strain extracts of Em A and Em a.

PLATE XXXVI.



DISCUSSION

The hypothesis that differentiation, development and function of vegetative and sexual organs of plants depend upon the action of specific chemical substances was advanced by Sachs more than 70 years ago. From that time onwards the progress in this area of developmental genetics has been progressing at a snail's pace and consequently not much information regarding the physiology of sexual reproduction has been brought to light.

In Neurospora the existence of a hormonal mechanism in the initiation and progression of sexual stages has been a debatable topic.

From complementation data and intergenic recombination of sterility mutants Vigfusson (1969) deduced that at least four genes control the 'early' stages of the sexual cycle in Neurospora while a minimum of three genes are indicated for those mutants blocking sexual development at later stages. The present study was designed to inquire into the existence of hormonal control of sexual development through the use of some of Vigfusson's sterility mutants. In addition, an attempt was made to investigate the existence of possible biochemical differences between the two mating types, A and a of Neurospora.

Vigfusson (loc. cit.) as well as Weijer and Yang (1963) reported that initiation of ascogenous hyphae is characteristic for both mating types of all N. crassa strains regardless of genetic background. Moreover, the incidence of ascogenous hyphae per unit surface growth is the same for both mating types of the same genetic background when grown under controlled conditions. Difference in fruiting abilities of different

strains is however common. The Emerson strains used in the present work are known to be less fertile than standard genetic strains such as the St. Lawrence strains. The reason for the use of Emerson strains for the present investigation is a historical one. Moreau and Moruzi (1931) in their first report concerning "sex hormones" in Neurospora made use of Emerson strains.

The technique adopted in this investigation employs crosses for testing the biological effect of the different extracts on the fertility of these crosses. Although in retrospect, there appears to be some merit in testing reciprocal crosses, one has to bear in mind that even in reciprocal crosses, the establishment and identification of the proto-perithecial parent is by no means accurate. Slight difference in the time of sexual differentiation of the individual strains may cause one parent to function inadvertently as the protoperithecial parent and the other as the spermatial one. It is for this reason that the reciprocal crossing technique was not employed in the present study and instead use was made of the technique of simultaneous inoculation of both mating types. Consequently, the effect of extracts on crosses has been expressed as an effect on fertility of the cross without reference to their effect on the individual protoperithecial and spermatial strain. The crossing technique of simultaneous inoculation of both mating types ensures that both mating types constituting the cross to be tested, are exposed to the extract treatment for the same time interval.

The sterile biochemical mutants tested for the biological activity of the extracts (Em A x Em a, Em A and Em a) were 5366-A, 7232-A, 10710-A, 9312-A, 10402-A and 8455-a. Of these, 5366-A, 7232-A and 10710-A

are 'early' mutants (i.e. the genetic block is believed to be situated before the association of male and female nuclei), whereas 9312-A and 10402-A are known to be 'late' mutants with a genetic block just prior to karyogamy or immediately after karyogamy (Vigfusson, 1969). The location of the genetic block of strain 8455-a is not known.

The extracts (Em A x Em a, Em A and Em a) proved to have a positive biological effect on the enhancement of the number of protoperithecia and/or on the improvement of fertility of all mutants tested with mating type A. The extracts did not have any effect on the number of protoperithecia to be initiated, or on the improvement of fertility of a sterile mutant of mating type a (8455-a, semi-sterile).

In the case of strain 5366-A the extracts (Em A x Em a, Em A and Em a) increased the number of protoperithecia initiated in a cross with Em a, but no restoration of fertility was noted. In all other cases (7232-A, 10710-A, 9312-A and 10402-A) there was some degree of improvement of fertility along with an increase in the number of protoperithecia initiated in a cross with Em a in the presence of extracts (Plate XII - XVII, XXIII - XXIX). Vigfusson (1969, p. 82) in his genetical study concerning sterility mutants reported that on the basis of complementation tests the mutant 5366-A falls in the first cistron of the 'early' genes. He postulated that the early steps in the sexual cycle involve: (a) production of diffusible substance(s) by the conidium; (b) attachment of the conidium by the trichogyne; (c) dissolution of the cell-walls of the conidium and trichogyne; (d) entry of the conidial cytoplasm and nuclei into the trichogyne; (e) migration of the male nucleus (nuclei) to the ascogonium and (f) association of the male and female nuclei in the ascogonium. As

the extracts are not effective in terms of restoration of fertility when used on mutant 5366-A, the present author postulates that some early steps in the sexual cycle (plasmogamy) are not controlled by the hormonal mechanism as present in the extracts, but possibly by different biologically active substance(s) or none. Although Backus (1939) reported hormonal control of trichogynal attraction by conidia (which can be considered as an early step in plasmogamy), Vigfusson's study does not substantiate this claim.

The extracts showed a prominent effect on two 'late' mutants (9312-A and 10402-A); in both mutants improvement of fertility was noted. The effect on the remaining two 'early' mutants (7232-A and 10710-A) was considered to be marginal. Only a few fertile perithecia in the mating with Em a and in the presence of the extracts (Em A x Em a, Em A and Em a). According to Vigfusson (loc. cit.) these latter two mutants constitute mutations of the fourth gene which controls the 'early' development of the sexual cycle (prior to plasmogamy). It seems pausible, therefore, that the extracts are more active on the later stages of sexual development (i.e. from karyogamy onwards), than on the early developmental sequelae.

Although genetic control of sequential progression of the sexual cycle has been extensively analysed in three Ascomycetes, e.g., Sordaria macrospora (Esser and Straub, 1956, 1958); Sordaria fimicola (Olive, 1956); and Glomerella cingulata, (Wheeler and McGahen, 1952; Wheeler, 1954), physiological details of these control systems have so far not been described. There exists scattered evidence in literature that physiological control in sex development is mediated by diffusible substances. As early as 1949 Markert concluded that fertility between

different strains of Glomerella was controlled by complementary factors and that these factors exerted their influence (in part at least) by means of a diffusible substance. Driver and Wheeler (1955) later demonstrated that a culture filtrate from a wild type stock induced perithecial production when added to a self-sterile culture. Esser and Straub (1958) observed the same phenomenon of selfing in Sordaria macrospora, viz., the occurence of selfed perithecia of one or both parents in crosses of self-sterile but cross-fertile strains. However, no diffusible agents could be found in the medium and, therefore, it was concluded that intracellular induction gave rise to 'selfing'. Vigfusson (1969), for the time, attempted to reveal by genetical methods the genes responsible for the development of the sexual cycle in the heterothallic Ascomycete Neurospora crassa. The present study constitutes the first attempt to uncover physiological details (hormonal control) of morphogenetic steps of sexual development both for homothallic as well as heterothallic Ascomycetes. The present study also attempts to elucidate the biochemical difference of the two mating types A and a of Neurospora crassa. The mating types in Neurospora are stable, controlled by an incompatibility mechanism and there is no report as yet of a mutation of one mating type to the other. Although both the mating types (A and a) are morphologically alike there is a genic difference between them with the ultimate outcome of a biochemical control of the two mating types through incompatibility mechanisms.

From early studies by Dodge and also by Lindegren it is evident that the sequence of morphological development steps with regard to sex in mating type A, is identical to those occurring in mating type a. The present data indicate that in mating type A these steps are under hormonal

control whereas such hormonal control seems to be absent for the control of sex development in mating type a: the extracts (Em A x Em a, Em A and Em a) were found not be improve the fertility of a mutant of mating type a (8455-a, semi-sterile). Although this observation involved only one mutant (8455-a), the conclusion derived from it is in agreement with the fact that the extracts (Em A x Em a, Em A and Em a) are not effective on the single strain of mating type a (see p. 88). The conclusion, however, that the sequelae of sex development (i.e. the ability to initiate protoperithecia and to establish sex heterokaryons) in mating type a is not genetically controlled remains totally unacceptable and it is for this reason that the present author is inclined to accept the possibility that the development of sex in mating type a involves a different gene product(s) (for which the extracts employed are lacking) than for sex development in mating type A. The present extraction procedures may not be efficient for the isolation of this substance(s). An alternative explanation concerns the possible existence of different threshold values of sex-inducing substances for the induction of sex development by the two different mating types. The present data with regard to the effect of different concentrations of the extracts on mating type a, are not indicative for such a mechanism.

In view of this conclusion, it appears that genetically determined heterothallism (as it occurs in N. crassa) gives rise to physiological heterothallism resulting from the differences in gene products as produced by the individual mating types.

The addition of extracts (Em A x Em a, Em A and Em a) yielded an increase in the number of protoperithecia initiated by the single

strain Em A, but not by Em a. Consequently, this observation is in agreement with the view that the induction of sex organs (protoperithecia) in the different mating types is controlled by different gene products, or in other words, that the strain specificity of the extracts in their biological activity is consistent with the qualitative nature of different sex-inducing hormones. The hypothesis that a diffusible substance from one mating type will have per se an effect on the opposite mating type (i.e., in the induction of sex organs), as is the case in some Phycomycetes (Achlya, Mucor, Allomyces), does not seem to be valid for the Ascomycete N. crassa. In the present study all three extracts - (cross Em A x Em a, Em A and Em a) were found to have positive effects on the number of protoperithecia initiated by strain Em A but not by Em a, although the extract of Em A was found to be less potent than the other two extracts. As outlined above for the effect of extracts from fertile crosses of Em A x Em a, experiments with extracts from single strains (Em A and Em a) are also indicative of the existence of a different gene product necessary for the development of protoperithecia by the a mating types, when compared with mating type A.

When the bisexual self-sterile strain (Isolate #I-28, Table IV) was used as a tester strain for the biological activity of the cross extract (Em A x Em a) a suppression in the initiation of protoperithecia was noted in the plates treated with the extract, when compared with untreated controls (Table V; Plates VIII - IX). Similar results were obtained with a hist-2A mutant of St. Lawrence genetic background. It is postulated here that the suppression of protoperithecia in the above mentioned strains is probably due to the fact that these strains produce the optimal quantity of sex inducing substance necessary for the

development of protoperithecia. An additional exogenous supply of hormone therefore will suppress the initiation of protoperithecia since the threshold value for optimal quantity has been exceeded. Since at present, no means are available to quantitate the biologically active substance, no attempt was made to verify this postulate.

Extract of the cross Em A x Em a induced 'selfing' in single strain cultures of Em A. Analysis of the progeny showed that segregation occurred for both the mating types (A and a), as well as for sterile and bisexual (self-sterile) isolates (Table IV). From a theoretical point of view this observation is very interesting in that it may indicate that the genome of mating type A is of a complex nature and includes the genome of mating type a. Relevant literature contains scattered reports of bisexuality in Neurospora. Lindegren (1934b) reported an ascus containing four bisexual and four akaryotic spores, the mycelium from each of the bisexual spores being self-fertile. The same author (1934a, 1936) described a bisexual, heterokaryotic, self-sterile strain in which self-fertility could be induced by a cross with a third highly fertile strain. St. Lawrence (see Olive, 1958) also obtained bisexuals in N. crassa but these were found to be self-sterile, indicating an incompatibility block leading to self-sterility. Moreau (1937) reported on a case of 'selfing' in N. tetrasperma in a cross of N. tetrasperma with N. sitophila. Weijer and Yang (1966) reported a self-sterile bisexual isolate with a meiotic segregation pattern suggesting the presence of a hybrid section of DNA with third division segregation in the ascus yielding bisexuals, 'A' and 'a' in the ratio of 1:3:4. Somatic segregation of these bisexuals yielded conidia of four mating type reactions: bisexual, 'A', 'a' and neutral. The explanation offered by these workers constituted an extension of the

polaron hybrid DNA theory to include somatic as well as meiotic recombination. A compound structure of the mating type locus was anticipated on the basis of these results. Other reports of bisexuality have been explained on the basis of chromosomal aberrations (Newmeyer, 1965) and disomy (Martin, 1959). The present results with regards to 'selfing' of Em A (as induced by cross extract) seem to support the concept of a compound structure of the locus of mating type A. Unfortunately, in the present study the necessary confirmation of the observation of selfing is lacking. Induction studies on a single strain, morphological (albino-A) as well as biochemical (hist-2A) mutant, yielded no perithecia.

Reports of 'selfing' as well as bisexuality in heterothallic species other than Neurospora can be found in literature. Esser and Straub (1958) found the occurence of 'selfed' perithecia of one or both parents in crosses of self-sterile but cross-fertile strains of Sordaria macrospora. Bistis (1956) whilst working with Ascobolus, obtained from a single spore isolate a self-fertile (bisexual) culture which gave rise to A and a progeny in one and the same tetrad. The vegetative growth (mycelium) of the bisexual strain after several transfers became a. A similar observation was made by Weijer and Yang (1966) in N. crassa. Nelson (1957) reported a self-fertile, single ascospore culture of Cochliobolus heterostrophus which retained its fertility after repeated transfers, but the ascospores of which produced heterothallic progeny (see Olive, 1958). According to Olive (loc. cit.) these ascospores either represent heterokaryotic structures or contain disomic nuclei carrying a homologous pair of compatibility chromosomes.

Homokaryotic fruiting may constitute a short-circuiting of the elaborate incompatibility system that results in functional homothallism. "Adequate analyses of this phenomenon are non-existent, but in at least one species, Schizophyllum commune, such fruiting appears to have extremely limited biological utility (Raper and Krongelb, 1958). Homokaryotic fruiting is sporadic, and production of spores is meager and the viability of spores is very low. Homokaryotic fruits have not been reported in nature and they appear in laboratory cultures only after prolonged sub-culture. The process is many orders of magnitude less effective than the normal dikaryotic production of spores and is considered, at least in this species, a derived - probably mutative - anomaly." (Raper, 1960, p. 798).

Vigfusson (1969) reported that the mutation frequency of the A mating type with respect to male sterility is more than 5 times higher than that of the a mating type (page 71). Unknown evolutionary inherent differences together with additionally unknown heterogeneities between the two mating types of N. crassa have been advanced by Vigfusson for the explanation of these results. Theoretically, on the basis of a more elaborate genetic structure of the A mating type locus (when compared with the locus of mating type a) a higher incidence of mutation for the A mating type can be expected.

On several occasions physiological differences between the two mating types of N. crassa have been reported (Fox and Gray, 1950a) and have been explained on the basis of the existence of different mating type alleles. Although the difference in mating type alleles contributes to physiological differences between mating types, as Fox and Gray (1950b)

were able to show, other genetic differences (other than these located in the mating type loci) are also involved. The present results seem to corroborate their explanation in favour of multiple genetic differences between the two mating types of N. crassa. In summary the observations (1) that a cross extract as well as single strain extracts are effective on A mating type strains (by increasing the number of protoperithecia developed by the tester strain) and not on a strains; (2) that the cross extract (Em A x Em a) induced 'selfing' of Em A (in one experiment) thereby giving rise to a progeny consisting of A, a, sterile and bisexual (self-sterile) isolates and (3) that cross as well as single strain improved fertility of A sterile mutants (except for 5366-A) and not of a a semi-sterile mutant (8455-a), import further evidence in favour of the existence of genetically determined physiological differences between the two mating types.

Raper (1960) in his statement defines sexual hormones as highly specific substances in origin as well as in function. Although the chemical substances isolated from N. crassa (and reported on in this thesis) enhance the development of protoperithecia, protoperithecia-like bodies and perithecia (when applied to test crosses), no evidence is available to show that such an enhancement is due to an extract induced increase in the initiation of sexual organs (ascogenous hyphae). Indeed, it is very well possible that the extract furthers the development of already present sex initials. The extract is however specific in that it improves fertility of "late" sterility mutants whereas an "early" sterility mutant remains completely sterile.

Although in literature these substances are referred to as 'sex hormones', the descriptive term of 'growth accessories' might in time prove to be more appropriate. In comparing Raper's (1960) definition of a hormone with others common in literature, its restrictiveness can be noted. Specificity of a hormone is usually limited to its action and to the minute amount necessary to bring about such an action rather than to the specificity of origin ('a chemical which is secreted by an endocrine gland in small amounts directly into the blood stream and greatly influences the functions of some specific organ and frequently of the body as a whole' - Dictionary of Bio-chemistry, Edit. W.M. Malisoff).

The restoration of fertility (by treatment with extracts) of one of the sterile isolates of the 'selfed' progeny of Em A is of considerable interest. The sterile isolate I-5 (Table IV) when crossed to Em a in the presence of extracts (Em A x Em a, Em A(?) and Em a) produced fertile perithecia whereas the control plates without extracts were devoid of such fruiting bodies (Plates VI, VII, XXX, XXXI, XXXII). Isolate I-5 in its vegetative behaviour showed, however, normal growth and normal development of sex organs (protoperithecia), and it appears, therefore, that this sterile mutant is either lacking the requisite quantity of the hormone necessary for successful mating or the particular hormone itself. An exogenous supply of a sufficient amount of this substance alleviated the block which underlies the sterility of this strain, thereby facilitating plasmogamy, karyogamy and the subsequent stages of ascosporogenesis.

The biochemical characterization of the compounds present in the extracts (Em A x Em a, Em A and Em a) as revealed by thin-layer chromotography and other methods, indicated that the cross extract

(Em A x Em a) contains two substances, whereas each single strain extract (Em A and Em a) contained only one substance.

Since extreme care was taken to preserve the integrity of the fungus during extraction procedures, the extract obtained is not a 'crude' extract but constitutes mainly an active secretional product or a degradation product of the cell wall together with contaminants of intra-cellular origin.

The physical appearance of the substances, their solubility in chloroform and 2:1 chloroform-methanol solvent (lipid specific) and the positive colour test with iodine vapour and bromothymol blue solution suggested the substances to be lipoid in nature. Due to the fact that the substances tend to run with the solvent front when polar solvents like benzene or 3:1 chloroform-benzene solution were used, suggested that the substances were hydrocarbon in nature. The non-mobility of the substances on thin-layer chromatography with non-polar solvent like hexane and carbon-tetrachloride indicated the substances to be unsaturated hydrocarbons. The absence of any carbonyl function in the infrared spectra of the extract of Em A and Em a pointed to either a straight chain or branched chain of saturated or unsaturated hydrocarbons. The mass-spectra seem to be in concurrence with the above suggested chemical nature of the compounds. The ultraviolet spectra of the extracts indicated a conjugated diene function (C=C-C=C), which of course is characteristic for the unsaturated nature of the compounds. Analytical combustion data showed the compounds to contain carbon and hydrogen only (C:85.01%, H:12.19% and C:85.62%, H:12.41%), with no appreciable amount of other additional elements The absence of any low field signals in the NMR spectrum indicated the absence of any olefinic

(C = C) or aldehydic ($-\underset{H}{C}=O$) or carboxylic ($-\underset{O-H}{C}=O$) configurations. Hence, all spectral analyses as well as analytical data of the extracts (and, therefore, of the biologically active compounds present in these extracts) agree on a chemical structure consisting of a long chain (straight or branched) of probably unsaturated hydrocarbons. At a later date, vapour phase chromatography employing suitable columns, may provide additional information with regard to the precise chemical identity of these biologically active compounds.

Both the compounds (either from Em A or from Em a) exhibited similar behaviour in thin-layer chromatograms and spectral analyses (UV, IR, NMR and Mass-spectra), although the molecular weights (as determined by the differential vapour pressure technique) of the biologically active substances in extracts of Em A and Em a differed to some extent. The molecular weight of the biologically active compound as present in the extract of Em a amounted to 354 to 372, whereas the compound from Em A yielded a molecular weight of 344 to 357. This small difference in molecular weights, together with other chemical data, seem to indicate that the two substances in question are closely related and probably differ in the length of the hydrocarbon chain.

Unfortunately, due to a lack of adequate amounts of the two individual compounds, biochemical identification of the extract from the cross Em A x Em a could not be extended beyond thin-layer chromatography.

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APPENDIX

i. The Effect of the Extract of Cross Em A x Em a on Em A

An interesting observation was made on a culture of Em A treated with different concentrations of the cross extract Em A x Em a. It was found that the tester strain Em A when treated with the extract in the concentrations of 0.1, 0.2, 0.3 ml/plate developed mature perithecia with spore content. Although the development of perithecia was slow, mature asci with ascospores were detected after six weeks of incubation. Three of these perithecia were dissected after three weeks of incubation and their content yielded ill-developed asci. After five weeks of incubation an additional two perithecia were opened and in one perithecium many asci with ill-developed ascospores and a few asci with mature ascospores were observed whereas in the other perithecium only five asci with ill-developed ascospores (predominantly abortive types together with a few giant types) were observed. Three more perithecia were dissected after six weeks of incubation and ascospores were found in two of them. Perithecia of the plates treated with 0.4 ml. and 0.5 ml. of extract were all found to be empty. Tetrad and random spore isolations were carried out in those cases where ascospores were found and in total 70 ascospores (39 random ascospores and 31 tetrad ascospores) were isolated. In total 25 ascospores (24 random ascospores and 1 tetrad ascospore) germinated. Mating type tests (Table IV) on these isolates indicated the following segregation for mating type:

A ---------- 8

a ----------10

Sterile--------- 6

Bisexual
(self-sterile)-- 1

Table - IV.

MATING TYPE ANALYSIS OF THE 'SELFED' PROGENY OF Em A

Isolate No.			Viability	Mating Type
Perithecia No.	Random or Tetrad	Spore		
I	Tetrad 1	1	-	
		2	-	
		3	-	
		4	-	
		5	-	
	Tetrad 2	1	-	
		2	-	
		3	-	
		4	-	
		5	-	
		6	-	
		7	-	
		8	-	
	Tetrad 3	1	-	
		2	-	
		3	-	
		4	-	
		5	-	
		6	-	
		7	-	
		8	-	
	Tetrad 4	1	-	
		2	+	Sterile
		3	-	
		4	-	
		5	-	
	Tetrad 5	1	-	
		2	-	
		3	-	
		4	-	
		5	-	

Table - IV (Continued)

Isolate No.			Viability	Mating Type
Perithecia No.	Random or Tetrad	Spore		
I	Random	1	+	.... A
		2	-	...
		3	-	
		4	+	.... a
		5	+	.... sterile
		6	+	.... a
		7	+	.... a
		8	-	
		9	+	.... A
		10	+	.... a
		11	+	.... a
		12	-	
		13	-	
		14	+	.... sterile
		15	-	
		16	-	
		17	+	.... A
		18	-	
		19	-	
		20	-	
		21	+	.... A
		22	+	.... sterile
		23	-	
		24	+	.... A (semi-sterile)
		25	-	
		26	-	
		27	-	
		28	+	Fertile with both mating types (bisexual)
		29	-	
		30	+	.... a

Table - IV (Continued)

Isolate No.			Viability	Mating Type
Perithecia No.	Random or Tetrad	Spore		
II	Random	1	+	.... a
		2	+	.... A
		3	+	.... a
		4	+	.... a
		5	+	.... A
		6	+	.... sterile
		7	+	.... a
		8	+	.... sterile
		9	+	.... A

N.B. Mating type tests were carried out in triplo.

ii. The Effect of the Extract of Cross Em A x Em a on Mutant Strain Albino-A

Since extract induced 'selfing' of an Em A strain produced spores of both mating types (Table IV), it was decided to test in a similar way a morphological mutant of N. crassa. In case 'selfing' could be induced in a morphological mutant it could be expected that all perithecia resulting from such a selfing should yield genetically identical offspring for this genetic marker. The mutant albino-A (mixed background) was grown in the presence of extract (7 replica plates per treatment) as well as without extract (control). It was observed, however, that the extract of the cross Em A x Em a had only a marginal effect on the number of protoperithecia initiated by the albino-A strain. A few, very small protoperithecia, developed during the incubation period and no ascospores were recovered. The experiment was repeated yielding similar results.

iii. The Effect of the Extract of Cross Em A x Em a on Mutant Strain Histidine - 2A

Subsequent to the results obtained with the albino-A strain, a biochemical mutant was selected for the induction of selfing by the extract of cross Em A x Em a. From a genetical point of view it could be expected that the offspring of the 'selfed' hist-2A would show no segregation for the biochemical marker and hence would be identical to the parental strain hist-2A irrespective of mating types. Since hist-2A has a St. Lawrence genetic background, it was decided to cross a hist-2a of Emerson background to Em A (as protoperithecial strain) in order to obtain a hist-2A mutant with an Emerson genetic background. A hist-2A mutant was recovered from this cross (hist-2a x Em A) and this isolate was grown with and without (control) the extract of Em A x Em a (20 replica plates per treatment). Plates treated with the extract developed many

visible protoperithecia after two weeks of incubation (Plate X). The control plates on the other hand, produced only a few (0-5) protoperithecia. After eight weeks of incubation well-developed protoperithecia were seen in some of the extract treated plates, however, no spore-shed was observed. Additional observations on these plates were also made after the tenth week of incubation. By this time the perithecium-like bodies had dried out completely and again no spore-shed was evident. On dissection, these fruiting bodies were found to be without ascospores.

In addition to the experiment above mentioned a hist-2A mutant but of St. Lawrence background was tested in a similar manner. On examination of the experiment, the control plates showed an increase in the number of protoperithecia when compared with the plates treated with the extract. Perithecium-like bodies were seen in the control as well as in the plates treated with the extract. No ascospores were observed. After five weeks of incubation 15 protoperithecia from the plates treated with the extract and 22 protoperithecia from control plates were dissected and were found to be void of ascospores.